ABSTRACT BOOK

NCI TRANSLATES

NCI Translational Science Meeting

November 7–9, 2008 • Grand Hyatt Washington Hotel • Washington, DC

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

1st NCI Translational Science Meeting

Welcome to the first NCI Translational Science Meeting. This event is an exciting opportunity to create a new forum to identify the most promising early laboratory research opportunities and move them rapidly and efficiently toward the clinic.

NCI has devoted considerable time and effort over the last 4 years to intensively studying how we conduct translational and clinical research. The Clinical Trials Working Group (CTWG) and its successor, the Translational Research Working Group (TRWG), defined a series of developmental pathways that are key to moving laboratory discoveries into early-stage clinical trials. Focused on realizing the promise of molecular oncology for patient and public benefit, the TRWG's first objective was to improve coordination and collaboration and instill a culture of active, goal-oriented management for the translational research enterprise. A second objective was to improve identification of the most promising early translational research opportunities across disease sites, populations, and pathways.

As we work to implement those recommendations, NCI is committed to becoming a more robust drug development resource for the cancer community—and to facilitating the vital collaborations from the public, private, and academic sectors that are necessary to make safe, efficacious multi-agent cancer therapies a reality for all of our patients. This meeting is certainly important for its science, but also as a symbol of our mutual commitment to progress in rapidly advancing—and changing—translational research.

It is my great hope that this meeting, with concurrent poster sessions and discussions, will help expand the range of collaborations and interactions among NCI-supported investigators and help us all better understand and put to use the CTWG and TRWG recommendations.

Most of all, this meeting is a chance to share outstanding science, to learn about each others' research, and ultimately to identify projects that we can move forward immediately. The success of this meeting will spring from the generous contribution of your time and participation. For that, I, along with all of my NCI colleagues, am most appreciative.

John E. Niederhuber, M.D.
Director
National Cancer Institute

DEPARTMENT OF HEALTH & HUMAN SERVICES

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November 7, 2008

Welcome to *NCI Translates: The NCI Translational Science Meeting*. This inaugural meeting brings together investigators from across all NCI-supported translational research mechanisms to share their expertise with the overall goal of accelerating translational cancer research.

This is NCI's first step towards implementing the Translational Research Working Group's (TRWG) recommended prioritization process. It will familiarize the research community with the TRWG Pathways to Clinical Goals, which serve as a guide for processes required to advance promising basic science discoveries to early-phase clinical trials.

During the poster discussion sessions, colleagues will share scientific expertise in order to identify collaborations that might advance discoveries to the clinic as rapidly, efficiently, and effectively as possible. The products arising from this meeting are expected to demonstrate the breadth, quality, and maturity of translational research opportunities, as well as the feasibility of applying the Pathways as a prioritization tool.

We thank you in advance for participating in this novel endeavor and for helping to transform translational cancer research.

On behalf of the NCI Translational Science Meeting Program Committee,

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NCI Translational Science Meeting

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NCI Translational Science Meeting Abstracts

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Presenting authors' names appear in bold, underlined text. (Note: changes may have been made after the abstract book was printed).
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001 Activation of Caspase-Independent Programmed Cell Death Overcomes Apoptosis Resistance in Ovarian Cancer Cells

Ayesha B. Alvero, Michele K. Montagna, Ki Hyung Kim, **Gil Mor**

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Introduction: Chemoresistance is a major burden in the treatment of patients with ovarian cancer and is associated with failure to induce apoptosis. In pan–resistant cancer cells, the full induction of the apoptotic cascade is inhibited due to high levels of expression of anti-apoptotic proteins, which prevent caspase activation. The objective of this study was to evaluate whether the activation of a caspase-independent alternative pathway could promote cell death in chemo-resistant ovarian cancer cells. We describe the characterization of a novel compound, NV128, which promotes cell death in a caspase-independent manner in chemo-resistant ovarian cancer cells.

Methods: Eight primary cultures and two established epithelial ovarian cancer (EOC) cell lines were treated with increasing concentrations of NV128 (0.1, 1, and 10 μg/ml) with or without the pan-caspase inhibitor, Z-VAD-FMK. Cell viability was determined after 24h using the Celltiter 96 assay. DNA fragmentation was analyzed by flow cytometry with Hoechst and Propidium iodide staining. Activity of caspases- 3/7, -8, and -9 was measured using Caspase-Glo assay. Protein expression was determined by Western blot analysis.

Results: NV128 treatment decreased cell viability in all tested EOC cells lines in a dose-dependent manner with IC_{50} between 1 and 5 μg/ml. Flow cytometry analysis revealed DNA fragmentation, with >90% cells staining double-positive for Hoechst and Propidium iodide after 24h. Cell death was however, caspase-independent as evidenced by the lack of caspases- 3/7, -8, and -9 activity and the inability of the pan-caspase inhibitor, Z-VAD-FMK, to prevent cell death. DNA fragmentation was observed as the result of the activation of an intracellular pathway involving: down-regulation of pAKT, cleavage of LC3 to LC3-II, Beclin mitochondrial translocation leading to Bcl2 inhibition, and nuclear translocation of EndoG.

Conclusion: We describe an alternative pathway leading to DNA fragmentation and cell death, which does not depend on caspase activation. Our findings demonstrate the possibility of using therapeutic drugs, such as NV128, which could overcome resistance to the classical caspase-dependent apoptosis and therefore have beneficial effects in chemo-resistant ovarian cancer patients.

Keywords: ovarian cancer, mTOR, caspase independent

002 A *Drosophila* Approach to Exploring Cancer Mechanisms and Therapeutics

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Cancer has proven a difficult disease to achieve significant long-term advances in patient survival; improvements in survival are often measured in months. Given this complexity, we have combined our approaches to take broader, whole animal approach to the complexities inherent in cancer and associated metastasis.

My laboratory has undertaken a genetic and drug screening approach targeting cancer and metastasis utilizing the fruit fly *Drosophila*. We have modeled several cancers including thyroid, breast, and lung. We have used these models to identify several pathways that mediate overgrowth and metastasis. Further, careful analysis of discrete tumors has suggested a novel model in which metastasis is the outcome of early, local interactions between normal and transformed epithelial cells specifically at tumor boundaries. We have worked with pathologists to identify evidence for this 'boundary effect' in human squamous cell carcinomas.

Using robotics, my laboratory has developed a method of high-throughput drug screening using robotics to screen *Drosophila* models of cancer and metastasis. This whole animal approach has helped identify a compound currently in Phase III clinical trials for Ret-based Medullary Thyroid Carcinoma and several candidate compounds that suppress metastasis in the fly and mouse. We are currently examining drug combinations and also screening for compounds that sidestep mutations that can lead to drug resistance. This whole animal approach holds promise to account for the inherent complexity of tumors in situ, which will aid in both exploring cancer mechanisms and identifying candidate therapeutics.

Keywords: Drosophila, MEN2, thyroid, breast, lung, drugs

003 Translational Development of Delta-24-RGD for the Treatment of Patients With Malignant Gliomas

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Malignant brain tumors are among the most deadly human cancers with a median survival of only one year. However, we have shown that Delta-24-RGD, a new, tumor-selective, infectivity-augmented, replication-competent oncolytic virus characterized by our group at M.D. Anderson may be an effective treatment against gliomas. Compared to other oncolytic adenoviruses, Delta-24-RGD is unique because its tumor selectivity is based on an alteration of the viral E1A gene, which renders it more selective and potent than previous oncolytic adenoviruses, and because of the addition of an RGD motif in the fiber knob, which increases its infectivity of tumor cells compared with normal cells. Indeed, preclinical studies have demonstrated the capacity of Delta-24-RGD to eradicate human gliomas in an animal model of the disease, and an IRB-approved Phase I clinical trial in patients with recurrent malignant gliomas (PI: FF Lang) is scheduled to begin in 2006 using GMP-grade agent, manufactured under the NIH RAID program (PI: WKA Yung/ J Fueyo). Despite the promising preclinical results supporting the Phase I trial, the ultimate success of Delta-24-RGD rests on its capacity to replicate in and spread throughout gliomas *in situ*, i.e. in patients. However, it is currently unclear the extent to which Delta-24-RGD is capable of producing oncolysis in tumors in patients. Consequently, a major goal of this project is to determine the extent to which Delta-24-RGD is capable of replicating in and spreading through human gliomas in situ. Although preclinical studies indicate successful viral replication and spread is likely to occur in patients' tumors, it is possible that for gliomas *in situ* complete tumor eradication (cure) may be limited at least partly by molecular impediments to viral oncolysis that reduce efficacy, and by complex physical barriers that will reduce the spread of virus from the site of injection to the edges of these infiltrative brain tumors. In this context, we have shown that the efficacy of Delta-24 is increased by combining it with specific chemotherapeutic agents, particularly Temozolomide (TMZ), a recently approved first line therapy for gliomas that is now considered standard of care. Interestingly, preliminary data indicate that Delta-24-RGD and TMZ have a synergistic anti-glioma effect, which is mediated at least in part by Delta-24-RGD-induced inhibition of MGMT and other DNA-repair pathways. Thus, the overall hypothesis of this project is that Delta-24-RGD will be efficacious in human glioma in patients specifically because of its capacity to replicate in and lyse tumor cells, and that the efficacy of the Delta-24-RGD can be improved by combining it with Temozolomide. In this grant application, we propose clinical and translational studies that by addressing our hypothesis will provide the fundamental information necessary for advancing this viral approach to useful clinical application.

Keywords: adenovirus, oncolytic viruses, E2F pathway

004 Anti-Angiogenic Therapy of Anaplastic Gliomas Using Thrombospondin Peptide and Radiation

Candece L. Gladson, L. Burt Nabors, Constance Y. Fears, Tanya A. Rege, **G. Yancey Gillespie**, Jack Henkin, Rod Humerickhouse

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The most frequent malignant brain tumors in adults are anaplastic gliomas, which are also the most lethal due to resistance to radiation and chemotherapy. These tumors are characterized by a substantial angiogenesis, inhibition of which is perceived to be an important strategy for limiting growth of these tumors. A number of natural molecules exist that are capable of inhibiting neovascularization. Among these, thrombospondin found in serum and some organs, and a small peptide derived from thrombospondin have been shown to induce apoptosis in microvascular endothelial cells that comprise these new blood vessels [Rege et al, 2005]. In the UAB Brain Tumor SPORE, Project #5, "Mechanism of Inhibition of Angiogenesis by Thrombospondin-1 and -2", Candece L. Gladson, M.D. and L. Burt Nabors, M.D. have determined that TSP-2 synthesized by host brain cells acted to inhibit angiogenesis in malignant gliomas [Fears et al., 2005]. Moreover, a thrombospondin peptide (CSVTCGDGVITRIR) was shown to induce apoptosis of proliferating brain microvascular endothelial cells by binding to CD36 and inducing proliferating endothelial cells to express Tumor Necrosis Factor-alpha (TNFα). Secreted TNFα was shown to ligate the TNF receptor and induce the cell death cascade. This novel *in vitro* observation was confirmed in two separate preclinical mouse brain tumor models in which the development of neovascularization and attendant growth of malignant gliomas in the brain was remarkably reduced by therapy with the thrombospondin peptide. In addition, these studies demonstrated that Src and focal adhesion kinase were necessary for anaplastic glioma proliferation *in vitro* and *in vivo* in mouse brain tumor models [Stettner etal, 2005; Ding et al., 2005].

These studies underpinned the rationale for a Phase I clinical trial using escalating doses of a peptide (ABT510) derived from Thrombospondin-1 together with standard radiation therapy (60Gy) to generate an anti-angiogenic effect. Twenty-four patients newly diagnosed with *glioblastoma multiforme* tumors received daily administration of ABT-510 during chemoradiation therapy, then daily thereafter. Doses were escalated in 4 successive cohorts of 3 patients each from 20 mg to 200 mg. An MTD was not defined. The last cohort was expanded by 12 patients for additional safety and response data. Response to therapy was assessed by MRI to determine changes in enhancing tumor volume. Time to tumor recurrence and overall patient survival times are being collected. Perfusion MRI was used to determine any changes in blood flow or blood volume in the tumor compared with contralateral uninvolved brain tissue. Tumor tissue initially resected was tested for the presence and amount of Thrombospondin-1 or -2.

Keywords: thrombospondin peptide, anti-angiogenesis, glioblastoma

005 Preclinical Development of SR13668, a Novel Dietary Indole Analog for Cancer Prevention and Therapy

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SR13668 (2,10-Dicarbethoxy-6-methoxy-5,7-dihydro-indolo[2,3-b]carbazole) is optimized from the in vivo active components of indole-3-carbinol, a naturally occurring anticancer agent commonly found in cruciferous vegetables. SR13668 exhibited potent oral anticancer activity against various human cancer xenografts in nude mice, including breast, prostate, lung and ovarian cancer. Its pharmacological profile is consistent with selective inhibition of phospho-Akt. SR13668 blocked EGF-stimulated Akt activation and inactivated its downstream effector GSK3β in PC-3 prostate cancer cells in a dose-dependent manner. SR13668 has no adverse effects on the fasting glucose levels or body weights after 14 days of oral treatment with SR13668 at 500mg/kg/day, a dose more than 50 times higher than the dose needed for antitumor activity. Screening a broad selection of 32 kinase targets, including Akt(1,2,3), PI3K, and PDK1, indicated that SR13668 is not a kinase inhibitor. SR13668 appears to inhibit Akt activation by blocking growth factor-stimulated Akt phosphorylation.

The drug development of SR13668 is supported by the NCI's Rapid Access to Preventive Intervention Development (RAPID) Program. Following a successful scale-up cGMP synthesis, SR13668 is being evaluated in standard toxicology studies to support Investigational New Drug (IND) filing. SR13668 did not show any liability in the standard genotoxicity battery testing (bacterial mutagenesis, in vitro chromosomal aberration, and in vivo mouse micronucleus). In a repeated 28-day oral dose toxicity study in male and female rats, the maximum tolerated dose (MTD) of SR13668 is considered to be greater than 1000 mg/kg/day based on the absence of drug-related mortality, morbidity, and organ toxicity at this dose level. Daily oral capsule administration of SR13668 at 30, 150, and 500 mg/kg for 14 days was well tolerated in both male and female beagle dogs. Cardiovascular safety pharmacology showed no changes attributed to SR13668 treatment. No dose-related toxicities or target organs of toxicity were identified by histological evaluation. Based on these findings, the NOAEL (no observable adverse effect level) in male and female beagle dogs is estimated to be above 500 mg/kg. Oral bioavailability of SR13668 at 30 mg/kg was approximately 20% in male rats and 25-40% in female rats. To optimize the bioavailability of SR13668 for clinical trials, study to evaluate different formulations under exploratory IND is in planning stages.

Keywords: Akt signaling pathway, indole-3-carbinol, cancer prevention and therapy

006 Ohio State Phase I U01 Program

Michael Grever, John Byrd, Thomas Lin, Guido Marcucci, William Blum, James Thomas, Miguel Villalona, Mitch Phelps

Ohio State University

The Ohio State University (OSU) is a comprehensive biomedical research campus with dedication to translational research in cancer therapeutics. The Phase I NCI/UO1 grant is strongly supported by the OSU Comprehensive Cancer Center and James Cancer Hospital. Basic and translational scientists from the respective Colleges of Pharmacy and Medicine who provide expertise in analytical, clinical, and molecular pharmacology supporting a multi-disciplinary team of phase I oncologists. This program has set itself apart by truly translating pre-clinical observations to phase I studies in hematologic malignancies, with subsequent follow through to phase II/III studies, while maintaining a robust solid tumor portfolio of novel single and combination agents. Areas of recent emphasis include CDK/signal transduction inhibitors, RNA-based therapies, epigenetic targeted therapies, and biologic (antibody/cytokine) based agents.

Recent drug development efforts emphasizing translational science include flavopiridol (Alvocidib), a cyclin dependent kinase inhibitor. Earlier phase I studies with this drug were disappointing, leading to its development being discontinued by the pharmaceutical sponsor. Investigators at OSU identified that differential protein binding between human and bovine serum had led to under-estimation of exposure required for effective killing of tumor cells. A phase I pharmacokinetic-directed study was designed at OSU to test the hypothesis that a plasma concentration of flavopiridol (1.5μM) level was required for 4-6 hours to achieve response. In fact, this schedule identified that flavopiridol was indeed highly active in CLL with a dose limiting toxicity of hyper-acute tumor lysis. Modification of the schedule of administration to improve safety was pursued, and we demonstrated that flavopiridol was highly effective in drug resistant, genetically high risk CLL. Additionally, we confirmed that response to flavopiridol correlated directly with pharmacologic endpoints. These promising results were confirmed in a separate phase II study, and this agent is now undergoing a registration trial by the sponsoring pharmaceutical company. This highly successful translational project identified that flavopiridol administered on the appropriate schedule is one of the most promising novel therapeutic agents in advanced CLL; our work has also energized enthusiasm for clinical exploration of this entire new class of cyclin dependent kinase inhibitors.

G3139 (Oblimersen Sodium) is a RNA-based anti-sense molecule targeting the 5'region of bcl-2. Investigators at OSU performed the first phase I study of G3139 and cytotoxic chemotherapy in acute myeloid leukemia (AML) and validated target modulation in a subset of patients at higher doses. A highly sensitive assay to quantify intracellular G3139 coupled with mRNA and protein determinations in leukemic cells confirmed that down-modulation of the target occurred proportionately to intracellular localization of the anti-sense. A subsequent phase III study of standard chemotherapy +/- G3139 failed to demonstrate therapeutic benefit for AML therapy, but the translational process from phase I through III was carefully executed to evaluate this molecular strategic approach in an effort to improve therapy for this fatal disease. Our current focus of study in AML is epigenetic targeting. We established a novel schedule of decitabine in AML based on a dose optimized for re-expression epigenetically silenced genes. Cooperation with the OSU U01 & N01 enabled a successful follow-up phase II study in elderly untreated AML.

Keywords: Phase I, U01, Flavopiridol

007 HotSpot™, a Novel Low Cost P33-based Kinase Assay for Kinomic Drug Discovery

Kurumi Y. Horiuchi, Sean Deacon, Yuan Wang,, Shuguang Liang, **Haiching Ma**

Reaction Biology Corporation

Protein kinases represent one of the most promising groups of drug targets due to their involvement in such pathological conditions as cancer, inflammatory diseases, neural disorders and metabolism problems. The plethora of different assay formats available today poses a great challenge to scientists who want to select a technology that will be cost efficient, convenient to use, and have low false positive and false negative rates. Unlike any other format kinase assay, the radioisotope based kinase assay has been considered as the "gold standard" for kinase assay. However, the cost and hazard associated with radioisotope usage have limited its development and commercialization. By developing new proprietary techniques and combining our expertise in nanoliter handling, we have developed a low cost 33P based kinase assay format, HotSpotTM, for routine HTS, qHTS and IC50 profiling service. Currently, more than 230 kinases have been developed and tested in this format. The Z factor for all kinases ranged from 0.7 to 0.9, ATP concentration can be performed from 1 uM to 500 uM. The new platform has made the kinomic approach an affordable reality for discovering inhibitors for a wide range of targets.

Keywords: kinase, cancer, profiling

008 A Multifaceted Program for the Development of Discodermolide: A Microtubule-Stabilizing Agent With Unique Additional Properties

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Discodermolide is a natural product that has the potential to be an important antitumor drug, particularly in Taxol resistant tumors. Although today there is an immense interest in 'targeted therapies' i.e. small molecules and antibodies that target aberrant signaling pathways in cancer cells, there is still a need for the development of new cytotoxic drugs that can be used independently and in combination with other cytotoxic agents and targeted therapies. Both Taxol and discodermolide are known to stabilize microtubules, which has profound effects on the replication of cells. The chemical structures of the two drugs are totally distinct, and although discodermolide has a mechanism of action with major similarities to that of Taxol, it also has distinct and intriguing differences. For example, 1) Taxol-resistant cells with mutations in β-Tubulin remain sensitive to discodermolide, 2) discodermolide cannot sustain the growth of Taxol-dependent cells, 3) discodermolide is a potent inducer of accelerated senescence, and 4) Taxol and discodermolide have a synergistic drug interaction. Our hypothesis is that discodermolide has a multi-modal, microtubule-dependent and –independent mechanism of action that may account for its unique properties relative to Taxol, and also may rationalize its potent synergy with Taxol. Our goal is to understand, at a molecular level, the mechanisms of action and of resistance to discodermolide in order to rationally develop the drug.

In a collaboration with Dr. Amos Smith, III, a structure-activity relationship (SAR) analysis of novel analogs of discodermolide is being undertaken in order to select active analogs that do not induce pulmonary toxicity, which has been reported in a single clinical study that was done with the drug. We believe that the decision to halt the clinical development of discodermolide was premature, with little attempt to understand the basis of its toxicities, or efforts to revert them. Our goal is to identify lead compounds for future pre-clinical and early clinical development.

Keywords: Taxol, discodermolide, microtubules.

009 Simultaneous Targeting of mTORC1 and the IGF-1 Receptor for Treatment of Childhood Sarcomas

Peter Houghton

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Insulin-like growth factors (IGFs) are implicated in the genesis and maintenance of childhood sarcomas such as Ewing sarcoma, rhabdomyosarcoma and osteosarcoma. These tumors are characterized by expression of the Type 1 IGF receptor (IGF-1R) and production of ligand, either IGF-1 or IGF-2. IGF's also protect against many forms of cellular stress, and prevent apoptosis induced by inhibition of mTORC1 signaling by rapamycin (1,2). Rapamycin treatment of sarcoma cells induces activation of the AKT survival pathway, in part by stabilizing IRS-1, and in part by stimulating secretion of IGF-1 in Ewing sarcoma cells. Consequently, we have examined the effect of combining an antibody that blocks ligand binding to IGF-1R (CP751871) with rapamycin. In vitro the combination of rapamycin with CP751871 significantly increased apoptosis compared to either single agent alone. Further, CP751871 and rapamycin had additive effects in suppressing VEGF secretion. To investigate the therapeutic potential, mice bearing advanced subcutaneous implants of childhood Ewing sarcoma (n=6), osteosarcoma (n=4) or rhabdomyosarcoma (n=2) were treated with CP751871 twice weekly for 4 weeks, rapamycin daily for 5 days for up to 12 consecutive weeks, or the combination. Whereas either single agent had limited antitumor activity in most models the combination was synergistic or supra-additive in 6 of 12 models. Rapamycin combined with CP751871 induced complete tumor regression in 1 Ewing, 1 rhabdomyosarcoma and 3 osteosarcoma models. Pharmacodynamic studies (day 1 and 7 of treatment) showed the similar inhibition of IGF-1R-mTORC1 signaling in responding versus progressing tumors thus did not track with tumor response. However, tumor-derived VEGF, determined by species-specific ELISA, indicated very significant suppression by the combination treatment in some of the responding tumors. These preclinical results suggest that combining agents that target IGF-1R and mTORC1 signaling may have significant antitumor activity against childhood sarcomas.

References: Thimmaiah KN et al., Cancer Res. 63:364, 2003; Huang S et al., Molecular Cell 11:1491, 2003.

Keywords: sarcomas, insulin-like growth factors, rapamycins

010 Akt, IKK and Erk, Three Oncogenic Kinases and Cancers

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FOXO3a is a transcriptional factor that was known to be involved in aging process. Akt was previously shown to inactivate FOXO3a. Later, we also demonstrated that IKK (***Cell*** 117:225-237, 2004), another oncokinase, induces tumorigenesis by inhibiting the Forkhead transcriptional factor, FOXO3a. It is known that Akt and IKK oncogenic kinases regulate nuclear exportation of FOXO3a, a forkhead transcriptional factor, by phosphorylation at different sites. More recently, we further demonstrated that Erk also interacts with FOXO3a and phosphorylates it in distinct sites recognized by Erk (***Nature Cell Biology*** 10:138-148*,* 2008). Erk-mediated FOXO3a phosphorylation increases FOXO3a cytoplasmic distribution and susceptibility to interaction and degradation by MDM2 that serves as a *bona fide* E3 ligase that mediates FOXO3a degradation. Thus, the three oncokinases, Akt, IKK and Erk that are frequently activated in human cancers, target at the common tumor suppressor., FOXO3a.

The tuberous sclerosis 1 (TSC1)/TSC2 tumor suppressor complex serves as a repressor of the mTOR pathway, and disruption of TSC1/TSC2 complex function may contribute to tumorigenesis. It is also reported that both Akt and Erk could target at TSC2 to induce tumorigenesis. In our most recent study (***Cell*** 130:440, 2007), it demonstrated that IKKβ, a major downstream kinase in the TNFα signaling pathway, physically interacts with and phosphorylates TSC1, resulting in suppression of TSC1. The IKKβ-mediated TSC1 suppression activates the TOR pathway, enhances angiogenesis, and results in tumor development. We further found that expression of activated IKKβ is associated with TSC1 Ser511 phosphorylation and VEGF production in multiple tumor types and correlates with poor clinical outcome of breast cancer patients. In summary, IKK could play an important role for inflammation induced cancer development by phosphorylating TSC1. The phosphorylation of TSC1 will activate the mTOR pathway, which is known to be involved in cancer development, thus the newly identified mechanism potentially links cancer, diabetes and obesity. Taken together, there are at least two common targets, FOXO3a and TSC complex associated with tumor suppression function, of three oncogenic kinases-Akt, IKK and Erk. The series of studies has led to novel discoveries of previously unknown signaling pathways/networks that play critical roles in cancer progression, which may serve as therapeutic targets for cancer intervention.

Keywords: TSC complex, FOXO3a, oncogenic kinases

011 Discovery of Anticancer Agents of Diverse Natural Origin

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The secondary metabolite constituents of tropical plants, aquatic cyanobacteria, and filamentous fungi are being investigated as part of a multi-disciplinary collaborative project directed towards the discovery of novel anticancer agents. Initial extracts prepared from these samples are evaluated in a variety of mechanistic and cell-based assays germane to cancer. Other aspects of this program project involve compound isolation, structure elucidation, dereplication, and *in vivo* biological testing using tumor growth inhibition bioassays, as well as biostatistics and active lead prioritization for potential further development. Examples of isolated compounds of recent interest will be presented. This collaboration involves The Ohio State University (Columbus, OH), the University of Illinois at Chicago (Chicago, IL), Research Triangle Institute (Research Triangle Park, NC), and the Pharmaceutical Research Institute, Bristol-Myers Squibb (Princeton, NJ). (Funding of NCI/NIH grant P01 CA125066-01A1 is gratefully acknowledged).

Keywords: tropical plants, aquatic cyanobacteria, filamentous fungi

012 Cellular and *in Vivo* Activity of a New PI3K Inhibitor-PX866 for Treatment of Human Glioblastoma

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The phosphatidylinositol-3-kinase (PI-3-kinase)/PTEN/Akt pathway is a prerequisite for a wide spectrum of cancers, either via the acquisition of activating mutations in the PI3K catalytic subunit itself, or via loss of PTEN. In this respect class 1 PI3Ks represents well-validated molecules for targeted drug discovery. We demonstrate, herein, that a newly developed PI3K inhibitor PX866, a wortmannin analog with selectivity for p110 α, β and γ (ProlX Pharmaceuticals), effectively inhibits signaling through the PI3K/Akt cascade in a set of glioma cells inhibiting various PI3K signaling components such as Akt, p70S6K1, TSC-2, and pS6. PX866 produces growth inhibitory activities in glioma cell lines with IC50 ranging from 4-8uM. PX866 did not induce apoptotic cell death where as it did induce autophagy- type-2 programmed cell death in U87 glioma cells in a dose dependent manner. In vivo experiment with U87 SC xenograft demonstrated an 84% growth inhibition after 4 weeks treatment at an oral dose of 2.5mg/kg in a qod schedule. PX-866 increased the median survival of animals with i.c.U87 tumors from 31 to 38 days with inhibition of p-AKT and p-S6 as shown by IHC and reverse phase lysate array. To develop a non-invasive method to assess biological activity of PX866, magnetic resonance spectroscopy (MRS) was performed to determine molecular response to PX-866 in the U87 model and tumor volume was assessed by T2 MRI sequence. Tumor volumes dropped on average from 20±11 mm^3 in control animals (n=10) to 5±4 mm^3 in PX-866 treated animals (n=10, $p<0.002$). To assess the potential of MRS-detectable metabolic changes as noninvasive biomarkers of response to PX-866, we performed localized MRS in control and treated animals. Spectra obtained from normal contra-lateral brain differed from spectra of untreated tumors in choline to NAA (Cho/NAA) ratio. Cho/NAA ratio was 0.8±0.1 in contra-lateral brain and increased to 1.7±0.4 (n=10; $p<0.002$) in the untreated gliomas. PX-886 treatment did not affect the spectra recorded from normal brain. In contrast, spectra from the tumor region indicated that Cho/NAA in PX-866-treated tumors was significantly lower by 30% compared to untreated tumors at 1.2±0.5 (n=10; $p<0.02$). Our findings demonstrate that PX-866-treatment results in MRS-detectable metabolic changes indicating a partial normalization of the metabolic profile of treated tumors. Taken together, these data demonstrate that PI3K inhibitor PX866 has significant activity in signal inhibition, cell cycle arrest, growth inhibition and autophagy in human glioblastoma in vitro and in vivo. In addition MRS can be used to non-invasively monitor the molecular effect of PX-866 in gliomas *in vivo* which further confirm the value of MRS in monitoring early molecular response to this PI3K inhibitor in patients *in vivo* affirming that PI3K/Akt pathway is a highly specific molecular target for molecular therapeutics development for glioblastoma and other cancers with aberrant PTEN/PI3K expression.

Keywords: PI3K inhibitor, glioblastoma, imaging

013 Plant-Derived Natural Product Analogs as Anticancer Clinical Trials Agents

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New therapeutic agents with diverse structures and mechanism of action have and will continuously be discovered from plant-derived natural products. Correspondingly, Dr. Lee's Natural Products Research Laboratories (NPRL) have discovered more than 2,000 novel cytotoxic antitumor natural products and synthetic analogs during over three decades of support from NIH Grant No. CA-17625 entitled "Plant Antitumor Agents". These discoveries are being used as new leads to develop future anticancer agents. The NPRL is also currently engaged in a NIH Roadmap Initiative Program for identifying active hits, which can serve as templates for new drug design, as well as avenues for better understanding of biological processes. Research in the NPRL previously led to the discovery of GL-331, a synthetic etoposide analog, which was in Phase II clinical trials as an anticancer drug. More recently, JC-9, a novel curcumin analog developed in the NPRL, was found to be a potent anti-androgenic agent. Planning is in progress to pursue clinical trials with JC-9 for prostate cancer in the near future, and it currently has succeeded in a Phase II clinical trial for treating acne. In addition, synthetic analogs of tylophorine and neotanshinlactone have shown efficacy in both cell and animal models and continue to be promising in preclinical studies as new drugs for treating lung and breast cancers, respectively. (Aided by CA17625 awarded to K. H. Lee.)

Keywords: natural products, plant antitumor agents, anticancer clinical trials agents

014 Development of ML-970 as a Potential Anticancer Drug

Moses Lee

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ML-970, NSC D76197, was designed as a novel analog of the highly cytotoxic DNA minor groove alkylating agents CC-1065 and the duocarmycins, as well as the clinically tested analogs adozelesin, carzelesin, bizelesin, and KW-2189. The latter compounds are highly potent cytotoxic agents and they demonstrated activity in the clinical studies. However, the use of these compounds is severely limited by their toxicity to the bone marrow. Another potential challenge in developing this class of compounds is the presence of a stereocenter in the molecules, which has been demonstrated to affect the DNA reactivity and cytotoxic potency. These limitations provided a rationale for the design, synthesis, and testing of new analogs with two specific goals: (1) Develop analogs that retain strong cytotoxic potency but with significantly reduced myelotoxicity, and (2) to eliminate the stereocenter, which should simplify the chemistry with respect to drug discovery and ultimately improve drug synthesis.

Through an active collaboration with the NCI Developmental Therapeutics Program and working through the Drug Synthesis & Chemistry Branch, more than 30 compounds from my laboratory were screened and ML-970 was discovered to have potent cytotoxicity in screening against a panel of 60 cell lines and also received positive results from the Hollow Fiber assay. Through further collaborative research with the Department of Oncology at University College London Medical School and with the Taiho Pharmaceutical Company of Japan, the DNA AT-sequence specific alkylation, DNA repair, and mechanism of action were examined. Concurrently, while in-vivo anticancer studies were conducted against mouse models (L1210 leukemia and B16 melanoma) in our laboratories, Taiho and the NCI performed in-vivo studies on human cancers grown as xenografts in skid mice. Significant activity against human cancer xenografts: lung, ovarian, breast, and CNS were observed when the compounds was administered via i.v., i.p. or p.o. Subsequent bone marrow toxicity studies showed that ML-970 was less toxic to both human and murine stem cells.

ML-970 represents a promising novel anticancer agent and this is a unique opportunity to take an agent with this mechanism into the clinic. Current studies are aimed at examining the pharmacokinetics properties of ML-970 when administered via the three different routes. In addition, through collaborations with the NCI and Spirogen, Ltd (UK), further *in-vivo* work, mechanism of action studies plus schedule, route and dosing studies are ongoing in an effort to improve on the activity seen in the initial xenografts. Results from these experiments will be presented and discussed in the presentation.

Keywords: minor groove, DNA alkylation, orally active

015 Development of an *in Vivo* Model to Study the Role of TrkB in Tumor Biology and to Develop Novel Therapies Targeting Activated TrkB

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Background: BDNF and TrkB are expressed in unfavorable, metastatic and highly angiogenic chemo-resistant NB tumors. Activation of TrkB-tyrosine kinase by BDNF protects NB cells from toxic effects of chemotherapy via PI3K/Akt pathway and stimulates metastasis and VEGF production *in vitro*. The aim of this study has been to develop a mouse model to 1) assess the effects of TrkB on NB tumor biology and 2) evaluate the efficacy of combining small molecule inhibitors of the TrkB/PI-3 kinase/Akt pathway with cytotoxic drugs in order to develop more effective less toxic therapy for poor prognosis NB patients.

Methods: Using tetracycline (Tet)-regulated TrkB-expressing NB cell lines, cells are treated with cytotoxic drugs in the presence or absence of BDNF. Prior to BDNF administration, cells are pretreated with inhibitors of the TrkB/PI-3kinase/Akt pathway followed by ETOP. Real-Time Cell Electronic Sensing system (RT-CES system) and MTS assays are used to detect the kinetics and extent of cell survival. To develop a mouse model, NB cells are injected at subcutaneous or orthotopic (periadrenal) sites in mice. Mice received water with or without Tet. When subcutaneous tumors reached 250-300mm^3, mice are randomized to receive etoposide(ETOP) alone or in combination with perifosine.

Results: *In vitro*, TrkB expression in cells cultured without Tet was 3.7 times higher than the TrkB expression in cells cultured with Tet. BDNF activation of TrkB caused up to a 50% reduction in the cytotoxic effects of chemotherapeutic drugs, such as ETOP, cisplatin and vinblastine. Pretreatment of NB cells with perifosine, Akt inhibitor, before BDNF administration blocked the BDNF-induced phosphorylation of Akt. Perifosine sensitized NB cells to ETOP, and partially blocked the BDNF protection of NB cells from ETOP-induced cell death. *In vivo*, TrkB expression in tumors from either subcutaneous or orthotopic sites in mice without Tet in water was increased 3-fold compared to TrkB levels in tumors from mice with Tet in water. Mice with subcutaneous tumors were treated with ETOP (10mg/kg or 20mg/kg). There was a 40% reduction in tumor size in low TrkB expressing tumors with 10mg/kg ETOP, while the tumors expressing high levels of TrkB required 20mg/kg ETOP to cause a comparable reduction. Yet if mice with high TrkB-tumors received daily doses of perifosine (24mg/kg), the tumor size was reduced by 40% with 10mg/ml ETOP. At this concentration, Perifosine alone had no effect on tumor size. These studies reveal *in vivo*, TrkB expressing NB cells are more resistant to cytotoxic therapy.

Conclusions: The biology of the NB tumors expressing high levels of TrkB in the *in vivo* mouse model supports findings in our *in vitro* models. The finding that high TrkB expressing tumors are more resistant to chemotherapy than low TrkB tumors raises the possibility that TrkB can be used as a biological marker of drug resistance. Our TrkB animal model will enable inhibitors of the TrkB/PI-3 kinase/Akt pathway to be screened to identify those that most effectively synergize with standard cytotoxics.

Keywords: TrkB, AKT, drug resistance, perifosine

016 Molecular Targeting Approach for Development of Antiviral Therapy of HIV Infection

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The development of antiretroviral therapy for AIDS has traced one of the most dramatic progressions in the history of medicine, showing the combinations of rapid drug development, short-lived trends, and continuous evolution. Upon the discovery of the first human pathogenic retrovirus HTLV-1, virtually no attempt was made to explore antiretroviral therapy, since it was believed that once target cells were infected by cellular-genome-integrating retrovirus and antiretroviral drugs would do nothing to the progress of the retrovirus-associated diseases. The first three dideoxynucleoside reverse transcriptase inhibitors (zidovudine or AZT, didanosine, and zalcitabine), developed at the National Cancer Institute, made changes. After these first drugs proved to be efficacious in patients with HIV infection, a number of antiviral agents were added to our armamentarium in the fight against HIV infection. The development of the second class of anti-HIV-1 agents, protease inhibitors, eventually produced sensational results in comparison to the clinical data that had been previously reported. Combination chemotherapy using such protease inhibitors with reverse transcriptase inhibitors has had a major impact on the morbidity and mortality of patients with HIV infection. However, we have faced multiple major problems, which represent the challenges different than we faced in the development of the first drugs. They include (i) drug-related toxicities, (ii) emergence of drug-resistant HIV variants, (iii) only partial restoration of immunologic functions, (iv) paradoxical flame-up of inflammation, and (v) increased cost of antiviral therapy. Nevertheless, extensive knowledge of the molecular, biochemical, and structural interactions of antiretroviral agents and their targeting viral components has been acquired. We are obviously at a new forefront in the therapy of HIV infection.

One new area in the development of antiretroviral agents is predictive modeling, which maximizes our chances of success. I will discuss an approach of combining site-directed mutagenesis-based data and molecular modeling, which represent a novel strategy for gaining structural insights for drug design of novel compounds. One of recently FDA-approved HIV therapeutic is darunavir, also developed at the NCI, was designed based on such structural approach. We most recently discovered that darunavir and a group of newly designed and synthesized agents block the dimerization process of HIV protease, an essential step in the replication cycle of HIV. Further improved approaches to explore new treatment modalities should be continued in the hope that with new and more potent antiviral agents, we will certainly be able to control HIV diseases more efficiently and effectively.

Keywords: AIDS, HIV, antiviral therapy

017 Apoptosis-Based Cancer Drug Discovery

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Defects in programmed cell death (apoptosis) play important roles in many aspects of tumor pathogenesis and progression, including chemoresistance and radioresistance, where apoptosis-resistance mechanisms impede successful eradication of cancers. The central theme of our drug discovery project (NCI-Drug Discovery Group, grant U19 CA113318) is to use knowledge of apoptosis targets and mechanisms for generating small-molecule drugs for the improved treatment of cancer. To achieve these goals, the project brings together the talents of accomplished molecular and cellular biologists, protein biochemists, structural biologists, and chemists into a collaborative effort involving three institutions located in close proximity (Burnham Institute for Medical Research, University of California at San Diego, and Torrey Pines Institute for Molecular Studies). By using a multidisciplinary approach that integrates medicinal chemistry, natural products, and combinatorial chemistry with modern drug discovery technologies encompassing advanced assay platforms and hit-to-lead optimizations, guided by structure- and NMR- based approaches, the research group has developed effective compounds that are currently at late-stage preclinical evaluations as anti-cancer agents.

Keywords: apoptosis, cancer, drug discovery

018 Development of Anticancer 1,2-Bis(sulfonyl)hydrazines

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We have designed and synthesized a new class of antineoplastic 1,2-bis(methylsulfonyl)-1-alkylhydrazine prodrugs (SHP) that are rapidly acting alkylating agents with $T_{1/2}$ activation values ranging from 10 s to 2.8 min depending upon structure. These agents owe their cytotoxicity primarily to the alkylation of the O6-position of guanine in DNA; the alkyl group donated is typically a methyl or a chloroethyl group. After O-6 chloroethylation of guanine, an intramolecular nucleophilic substitution reaction occurs generating N^1,O^6-ethanoguanine; this intermediate slowly reacts with the N-3 position of an opposing cytosine to form a 1-(N^3-deoxycytidinyl)-2-(N^1-deoxyguanosinyl)ethane (G-C) cross-link, which is extremely cytotoxic (< 10 lesions per cell can produce lethality). Replacement of the hydrogen in the N-2 position of the SHPs with masking groups prevents the reactions that leads to alkylation; the active alkylating species can be liberated by hydrolysis, thiolysis, glutathione S-transferases, enzymatic cleavage, or hypoxia selective reduction of a trigger/linker attached to the N-2 position. The moiety used for latentiation may be a secondary active species or be used to tune pharmacokinetics or both. O^6-Alkylguanine-DNA alkyltransferase (AGT) is the major repair mechanism for lesions of this type. AGT readily repairs O^6-methylguanine, O^6-(2-chloroethyl)guanine and N^1,O^6-ethanoguanine, but cannot repair the G-C cross-link. AGT activity can be present in tumors, resulting in resistance to SHPs, and in normal tissues, protecting them from the cytotoxicity of SHPs. Potent AGT inhibitors are known which greatly sensitize AGT expressing cells to SHPs. Cloretazine (1, 2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine), the initial SHP to enter clinical trial, has a $T_{1/2}$ of ≈ 1h at physiological pH and liberates two short-lived reactive species, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine, ($T_{1/2}$ ≈ 30 s, pH 7.4, 37°C), and methyl isocyanate, ($T_{1/2}$ ≈ 1.7 min, pH 7.4, 37°C). Cloretazine has shown broad anticancer activity in preclinical screens and significant activity in elderly acute myelogenous leukemia patients in Phase I and Phase II clinical trials.

A second SHP, 1, 2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine, designated KS119, with selective activation by hypoxic cells of solid tumors, is in preclinical development. This agent exploits the reductive potential of the hypoxic tumor cells to preferentially target hypoxic cells.

Keywords: targeted-alkylating agents, Cloretazine, KS119

019 Treating Advanced Cancer by Disrupting the Tumor Ecosystem

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Tumors can be defined as ecosystems in which the various host and cancer cells (species) interact with their environment. A key component of the tumor ecosystem is tumor associated macrophages (TAMs), which can be considered an invasive species within the tumor ecosystem that support the growth of the cancer cells. TAMs offer an attractive target for cancer therapeutics. We have identified monocyte chemoattractant protein – 1 (MCP-1, CCL2) as a novel and potent regulator of prostate cancer tumorigenesis because it is the key mediator of the attraction of TAMs to the tumor sites.

CCL2 is a member of the CC chemokine family and was originally described for its sentinel role in regulating monocyte / macrophage migration to sites of inflammation and wound repair. CCL2 has been shown to be an active mediator of tumorigenesis and metastasis of several cancers, including roles in regulating the migration and proliferation of breast, cervical, pancreatic, multiple myeloma, and prostate cancer cells. We have demonstrated:

- Utilizing tissue procured through the Prostate SPORE Rapid Autopsy Program at the University of Michigan, we determined that CCL2 was significantly overexpressed in bone metastases as compared to normal tissues as well as metastases from other sites.
- Analysis of CCL2 secretion by several constituents of the bone-tumor microenvironment by ELISA revealed that the bone marrow endothelial cells secrete significantly higher basal levels of CCL2 compared to PCa cells, osteoblasts, and adipocytes.
- Analysis of human tumors from metastases as well as from *in vivo* preclinical models reveals a high percentage of infiltrating macrophages.
- CCL2 stimulates the maturation of osteoclasts in the bone tumor microenvironment.
- Inhibition of CCL2 *in vivo* leads to significant inhibition of tumor growth in multiple preclinical models of prostate cancer and other cancers.

CCL2, therefore, mediates tumor growth through effects on cancer cell proliferation and migration, osteoclast maturation, and macrophage recruitment and education. Strategies that inhibit CCL2 are in active development and have entered into clinical trial. Proof of principle trials are planned through the SPORE mechanism. Because this type of therapy is targeted against various aspects of the microenvironment, trials across disease sites are planned through SWOG.

Keywords: therapy, prostate, monocyte

020 The Indenoisoquinolines Non-Camptothecin Topoisomerase I Inhibitors: Update and Perspectives

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Because camptothecins are effective against previously resistant tumors and are the only class of topoisomerase I (Top1) inhibitors approved for cancer treatment, we developed the indenoisoquinolines. Like camptothecins, the indenoisoquinolines selectively trap Top1-DNA cleavage complexes and have been co-crystallized with the Top1-DNA cleavage complexes. Indenoisoquinolines show antitumor activity in animal models. They have several advantages over the camptothecins: (1) They are synthetic and chemically stable (unlike camptothecins); (2) The Top1 cleavage sites trapped by the indenoisoquinolines have different genomic locations than camptothecins, implying differential targeting of cancer cell genomes; (3) The Top1 cleavage complexes trapped by indenoisoquinolines are more stable than for camptothecins, indicative of prolonged drug action; and (4) They are less or not substrates for the multidrug resistance efflux pumps (ABCG2 and MDR-1). Among the more than 400 indenoisoquinolines synthesized and evaluated, three have been retained as leads for clinical development by the NCI: NSC 706744, NSC 725776 (Indimitecan) and NSC 724998 (Indotecan). The trapping of Top1 cleavage complexes by indenoisoquinolines in cells results in the rapid and sustained phosphorylation of histone H2AX (referred to as γ-H2AX). We will also discuss the use of γH2AX as a pharmacodynamic biomarker for the clinical development of the indenoisoquinolines.

Keywords: topoisomerase, DNA, cancer treatment

021 Receptors and Transformation

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While the role of liganded nuclear receptors in mediating coactivator/corepressor exchange is well established, nuclear motor-dependent regulation of chromosomal organization in the three-dimensional space of the nucleus to diverse signaling events is emerging as a major parallel strategy to achieve integrated transcriptional responses. Chromosomal translocations and their corresponding gene fusions play important roles in many diseases of development and in initial steps in cancers, which is particularly well established for leukemias, but now identified is a more general event in solid tumors. The molecular basis for these tumor translocation events is quite incompletely understood, although a prevailing model, instituted by events in yeast, is that random translocations are selected by conferring growth advantage. We can suggest a model for the molecular logic underlying tumor translocation events, linking these translocations to evolutionarily-conserved strategies, with the liganded nuclear receptor (AR) serving as a driver of non-random and tissue-specific chromosomal by inducing both the juxtaposition of the recombination sites, based on local chromatin modification, leading to a synergistic enhancement of DSB at those sites in response to genomic insults.

Keywords: nuclear receptors, chromosomal translocations, androgen receptors

022 Targeting Geranylgeranyltransferase I for Anti-Cancer Drug Therapy in Tumors with Hyper-Activated Ras / Ral GDS / Ral A/B and/or With Low p27Kip

Said M. Sebti

Drug Discovery Program, H. Lee Moffitt Cancer Center

The ability of many GTPases to contribute to malignant transformation requires their posttranslational modification with farnesyl or geranylgeranyl lipids. This prompted us and others to develop farnesyltransferase and geranylgeranyltransferase I inhibitors (FTIs and GGTIs) as novel anti-cancer drugs. Several FTIs have been developed and some reached clinical trial evaluations. However, GGTIs have not been evaluated in humans. In this poster we will present pre-clinical data supporting the use of our clinical candidate, GTI-2418, in patients whose tumors contain hyper-activated Ras / Ral GDS / Ral A/B pathways and/or low levels of the cyclin-dependent kinase inhibitor p27Kip. This work is supported by the NCI NCDDG grant U19 CA67771 and resulted from collaborations among its 3 programs from Moffitt (Sebti, Lawrence, Guida), Yale (Hamilton), UNC (Cox, Der, Sondek).

Keywords: Geranylgeranyltransferase, GTPases, Ral A/B, p27Kip

023 Targeting Signal Transduction Pathways for Cancer Drug Discovery

Said M. Sebti

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In human cancers many components of signal transduction pathways are hyper-activated including the phosphatase SHP2 and the GTPase Ras which activate the serine/threonine kinase Raf which in turn binds, phosphorylates and inactivates the tumor suppressor pRb. Other components of signal transduction that are aberrant are those that allow tumors to evade apoptosis and include inactivation of the tumor suppressor p53 by binding the oncoproteins mdm2 and mdmx, overexpression of the anti-apoptotic Bcl proteins, and sustained degradation of the proapoptotic proteins Bax and IKB by the proteasome. These aberrant signal transduction pathways are intimately involved in oncogenesis and have been associated with poor prognosis, resistance to chemotherapy and shortened patient survival time. The central hypothesis upon which our P01 program project is based is that disruption of mdm2/p53, mdmx/p53, Raf/Rb and Bcl/Bax associations and inhibition of SHP2 and proteasome activities will induce apoptosis and inhibit malignant transformation and tumor growth in human cancer cells. In this poster progress for each of the above 5 projects will be presented. This work is supported by NCI P01 grant CA 118210 which includes 5 programs and 3 cores lead by Drs. Chellappan, Chen, Guida, Lawrence, McLaughlin, Sebti, Wang, and Wu.

Keywords: SHP2, mdm2, p53, Rb, Raf, Bcl, Bax

024 Developing Withacnistin as a Novel Anti-Cancer drug in Tumors That Contain Persistently Activated STAT3

Said M. Sebti

Drug Discovery Program, H. Lee Moffitt Cancer Center

The signal transducer and activator of transcription 3 (STAT3) is found aberrantly activated in the majority of human cancers. We have identified from the NCI chemical libraries the natural product withacnistin as a potent inhibitor of STAT3 activation. Withacnistin is selective for STAT3 over other signal transduction pathways such as those mediated by MEK, AKT, JNK or p38. Withacnistin also inhibits the activation of other STAT family members such as STAT5 and STAT1. Withacnistin inhibits growth factor (EGF, PDGF) and cytokine (IL-6, IFN-beta, GM-CSF) STAT activation. Importantly, withacnistin inhibits tumor growth and induces apoptosis selectively in those tumors where STAT3 is persistently and aberrantly activated. Withacnistin is undergoing advanced preclinical studies under the NCI RAID program in preparation for translating our laboratory findings to the clinic. (RAID 135073)

Keywords: STAT3, withacnistin, apoptosis

025 Pharmacodynamic Assessments of Cyclin-Dependent Kinase Inhibitors: Modulation of Cdk Targets in Clinical Samples

Geoffrey I. Shapiro, Leonard J. Appleman, Delores DiVizio, Lucian R. Chirieac, Anat Stemmer-Rachamimov, Fionnuala O'Connell, Nandita Bhattacharya, Massimo Loda

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Cyclin-dependent kinases (cdks) play critical roles in cell cycle progression and RNA transcription. Cell cycle cdks, including cdks 2, 4 and 6, phosphorylate substrates including the retinoblastoma protein (Rb), $p27^{Kip1}$ and E2F-1 to facilitate G1 and S phase progression; inhibition of these cdks leads to both cell cycle arrest and apoptosis. Transcriptional cdks, including cdks 7 and 9, phosphorylate the C-terminal domain (CTD) of RNA polymerase II, and control efficient transcriptional initiation and elongation, respectively. Transcriptional cdk inhibition depletes cells of mRNAs and encoded proteins of short half-life, including cyclin D1, anti-apoptotic proteins, and Mdm2, the latter facilitating accumulation of p53. Several cdk inhibitors have entered clinical trials, including flavopiridol, seliciclib, BMS-387032/SNS-032 and others, with variable potencies against the cell cycle and transcriptional cdks. In patients with advanced solid tumors, prolonged stable disease has been documented, although objective responses are rare. Clinical trials have not routinely incorporated pharmacodynamic assessment of inhibition of the target kinases in patients. To determine whether these agents are indeed inhibiting cdks in either tumor or surrogate proliferating tissue, we have conducted pharmacodynamic studies in the context of Phase 1 trials in patients with advanced solid tumors.

Skin biopsies or tumor sampling were performed prior to and within 2 hours of completion of the first intravenous infusion of flavopiridol or other agents. Paraffin-embedded samples were analyzed by immunohistochemistry for effects of treatment on cdk targets including total and phospho-Rb (cdk2 and cdk4), total and phospho- p27 (cdk2) and p53 and cyclin D1 (cdk9). Approximately 200 nuclei were routinely scored for 0, 1+ and 2+ staining; the sum of 1+ and 2+ staining was used to determine the % positive cells. Additionally, in an ex-vivo, plasma-based assay, pre- and post-treatment plasma was applied to stimulated peripheral blood mononuclear cells (PBMCs) to assess effects on cellular proliferation.

In a Phase 1 trial of flavopiridol, using 1-hour infusions administered 3 weeks of every 4, 6 of 12 paired sets of skin biopsies demonstrated reduced staining of Rb [pS249/252] post treatment ($p = 0.025$) and 8 demonstrated decreased Rb staining at the [S807/811] phosphorylation site ($p < 0.002$). Depletion of phospho-Rb occurred while staining for total Rb was preserved. In addition, increases in total $p27^{Kip1}$ occurred post-flavopiridol, suggesting decreased [pT187] phosphorylation. Consistent with inhibition of cdk9, the majority of paired skin biopsies demonstrated either stable or increased p53 levels post-treatment. Reduced staining of cyclin D1 was also demonstrated. In two patients with accessible tumor tissue, staining demonstrated decreased phospho-$p27^{Kip1}$, increased total $p27^{Kip1}$ and decreased phospho-Rb [pS807/811] post treatment. In the context of second Phase 1 trial, in which a prolonged flavopiridol infusion was administered after gemcitabine, post-treatment plasma obtained from patients treated at the maximum tolerated dose reduced proliferation of stimulated PBMCs by at least 25% compared to pre-treatment plasma in 8 of 9 paired samples ($p < 0.0005$). Finally, in a trial of a more selective cdk2 inhibitor, increased $p27^{Kip1}$ occurred post-treatment among 7 of 7 paired skin biopsies ($p = .00133$). These data demonstrate that cdk inhibitors can induce biologic effects in tumor and surrogate proliferating tissue, and suggest these markers can be used in the phase 2 setting to correlate with clinical outcome.

References: Shapiro GI. J Clin Oncol 24: 1770, 2006; Haddad et al. Clin Cancer Res 10:4680, 2004; Zhang et al. Nat Med 10:643, 2004.

Keywords: cyclin-dependent kinase inhibitor, cell cycle, immunohistochemistry

026 Natural Product-Based Anticancer Drug Discovery: the National Cooperative Drug Discovery Group at University of Wisconsin-Madison

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Natural products have an excellent track record as sources of anticancer drugs. However, a natural product-based anticancer drug discovery and development program is often hampered by (1) the complex molecular architecture of natural products that renders chemical total synthesis and medicinal chemistry ineffective, limiting their accessibility for both mechanistic and clinical studies, (2) the unknown or poorly characterized molecular targets that natural products act on, and (3) the general dose-limiting toxicity due to lack of cancer cell-specificity.

The UW-Madison National Cooperative Drug Discovery Group (NCDDG) program builds on a carefully selected set of natural products primarily of microbial origin, which have proven cytotoxicity or antitumor activity, and aims to develop them into new forms ("lead analogs") suitable for development into clinically useful drugs. This will be accomplished by enhancing structural diversity, identifying novel targets, and delivering the natural product drugs to specific cancer cells, through the use of combinatorial biosynthesis and chemoenzymatic approaches. The compounds generated will be evaluated in vitro first to eliminate compounds with low activity, using biochemical assays and cytotoxicity determinations in tumor cell lines. Those compounds that meet our criteria for initial biological activity will be tested in vivo for antitumor activity in autochthonous mouse models of colon (due to mutation in APC), breast (due to overexpression of Wnt1), cervical (due to expression of human papilloma virus E6 and E7 genes altering p53 and Rb) and pancreatic (due to overexpression of c-myc) cancer to guide our anticancer drug analog discovery work.

The goal of the UW-Madison NCDDG program is to develop known cytotoxic and antitumor agents from natural sources into compounds that can lead to improved cancer drugs, using specialized biochemical and genetic methods that take advantage of recent advances in microbial genetics, cancer cell biology and animal models of important, difficult-to-treat human cancers. The approach we will take to the discovery and development of new treatments for cancer is tailored to the special advantages as well as difficulties of working with natural product drug leads. While leads obtained through screening natural sources may uncover entirely new structures that reveal new mechanisms by which the compounds thwart malignant cells from becoming preeminent in the body, a more expedient and certainly more predictable approach is to start with a natural product that has proven antitumor potential, then explore ways to develop it into a more effective drug. This is the hallmark of UW-Madison NCDDG program.

Keywords: natural product, national cooperative drug discovery group, autochthonous mouse model

027 Discovery of Novel Anticancer Agents From the Johns Hopkins Drug Library

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The discoveries of novel pharmacological activity of known drugs, sometimes referred to as the "off target" effects, have appeared in the literature from time to time. Recent years have witnessed an increase in frequency of the appearance of such discoveries. Finding new indications for existing drugs offers a number of advantages. First, since existing drugs are already in use in humans, they are bioavailable and have tolerable side effects. Second, the well-established toxicity and bioavailability of known drugs in humans will significant reduce the time and cost to evaluate drugs in the new indications in humans. Last but not least, the extensive knowledge on the mechanisms of action of known drugs and the availability of a large number of analogs during the development of each drug will significant facilitate the understanding of the molecular basis of the new pharmacological activity and enable rapid structure/activity relationship study to develop new generations of the drug in the context of the new indication. Together, the advantages of this approach call for a systematic effort to collect and screen available FDA-approved drugs for new pharmacological activities. We began a new initiative to systematically collect and assemble a library of clinical drugs in 2003. To date, we have to date collected over 2,400 existing drugs and assembled them into the Johns Hopkins Drug Library. We have screened this library in a number of cell-based assays for new anti-angiogenic and antitumor agents. Novel and unexpected hits have been identified in each of the screens performed. Mechanistic deconvolution of those hits has shed new light on the regulation of proliferation of endothelial and cancer cells. Moreover, some of the hits have been shown to be efficacious in blocking angiogenesis and tumor growth in vivo, suggesting that they are promising leads for development into novel anticancer drugs.

Keywords: drug library, screening, animal models

028 Targeting Cell Cycle and Checkpoint Pathways in Well-differentiated and Dedifferentiated Liposarcoma

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Liposarcoma is the most common type of soft tissue sarcoma, accounting for about 25% of all soft tissue sarcoma in adults. Classification of liposarcoma into three biological types encompassing five subtypes: (1) well-differentiated/dedifferentiated; (2) myxoid/round cell; and (3) pleomorphic, based on morphological features and cytogenetic aberrations, is widely accepted, however, diagnostic discordance remains even among expert sarcoma pathologists. The well-differentiated and dedifferentiated type most commonly occurs in the retroperitoneum and is characterized by 12q amplification with a diagnostic doublet of CDK4 and MDM2. While these tumors can be treated surgically, they frequently recur and there are few effective systemic therapies. By performing a comprehensive genomic analysis, we now have an opportunity to identify new therapeutic targets for this deadly disease. We sought to develop a more systematic approach to liposarcoma classification based on gene expression analysis and to identify subtype specific differentially expressed genes that may be involved in liposarcoma genesis/progression and serve as potential therapeutic targets. A classifier based on gene expression profiling was able to distinguish between liposarcoma subtypes, lipoma and normal fat samples. A 142 gene predictor of tissue class was derived to automatically determine the class of an independent validation set of lipomatous samples and demonstrates the feasibility of liposarcoma classification based entirely on gene expression monitoring. Differentially expressed genes for each liposarcoma subtype compared to normal fat were used to identify histology-specific candidate genes with an in-depth analysis of signaling pathways important to liposarcoma pathogenesis and progression in the well-differentiated/dedifferentiated subset. The activation of cell cycle and checkpoint pathways in well-differentiated/dedifferentiated liposarcoma provides two possible novel therapeutic strategies:

1) Targeting CDK4 and the G1/S cell cycle checkpoint with flavopiridol a pan CDK inhibitor. We demonstrate that CDK4 is consistently amplified and overexpressed in dedifferentiated liposarcoma (DDLS) compared to normal fat. We then show that DDLS cell lines with amplified CDK4 are susceptible to flavopiridol induced apoptosis and that DDLS xenografts are 2-fold more sensitive to flavopiridol than doxorubicin. This anti-tumor effect was enhanced when appropriately sequenced with doxorubicin. The transfection of CDK4 into a sarcoma cell line without amplified and over-expressed CDK4 results in a significant increase in sensitivity to the growth suppressive and apoptotic effects of flavopiridol. Collectively this data was used to initiate a Phase I trial of doxorubicin and flavopiridol in the treatment of metastatic sarcoma particularly targeting sarcoma subtypes with amplified CDK4. Twenty two patients have been treated on this trial(CTEP study #6204) and the MTD of flavopiridol is yet to be determined. We have observed 1 PR in a patient with metastatic leiomyosarcoma. We have also observed prolonged stable disease. This included a patient with liposarcoma who received her maximal dose of doxorubicin (600 mg/m2) with flavopiridol and then, per protocol, continued flavopiridol alone as a single agent every 3 weeks. This patient remained on study almost 2 years. This study continues to accrue patients.

2) MDM2 and the G2/M cell cycle checkpoint is a promising target in DDLS with amplified MDM2. We show that Nutlin-3a, an antagonist of MDM2, preferentially induces apoptosis and a G2/M growth arrest in DDLS cell lines compared to normal adipocytes. DDLS cells retain critical functionality in downstream p53-dependent apoptotic signaling pathways. These results support the development of a clinical trial with MDM2 antagonists for patients with recurrent well-differentiated/dedifferentiated liposarcoma and show the promise of this transcriptional dataset to provide additional novel targets for drug discovery.

Keywords: genomic analysis, sarcoma, cell cycle targets

029 Discovery and Development of Novel Small-Molecule Inhibitors of Bcl-2 and Bcl-xL Proteins as a New Class of Anticancer Drugs

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Intensive research in cancer biology has now firmly established that the impaired ability of cancer cells to undergo apoptosis plays a major role in the resistance of cancer cells to chemotherapy or radiation and for the failure of current anti-cancer drugs. Evasion of apoptosis is commonly recognized as a hallmark of all cancers. Hence, future efforts to design new, molecularly targeted, anticancer therapies aimed at improvement in the survival and quality of life of cancer patients must include novel strategies that specifically target the resistance of cancer cells to apoptosis. In the past decade, research into apoptosis has identified several classes of regulators of apoptosis. These critical apoptosis regulators provide important new molecular targets for anticancer drug design. The anti-apoptotic Bcl-2 family of proteins, including Bcl-2, Bcl-xL and Mcl-1, arguably represents one of the most exciting classes of molecular targets for design and development of an entirely new class of anti-cancer drugs for the following reasons.

Our NCDDG project aims at the design, synthesis and evaluation of novel nonpeptidic, small-molecule inhibitors of Bcl-2 and Bcl-xL proteins as a new class of anti-cancer drugs through a structure-based, contemporary, multidisciplinary and integrated drug discovery approach. Compared to the existing antisense oligonucleotide strategy targeting Bcl-2 and Bcl-xL, properly designed and optimized nonpeptidic small-molecule inhibitors of Bcl-2 and Bcl-xL should have several major advantages as drug molecules. These include improved cell-permeability, in vivo stability and bioavailability, ease of administration and lower cost. Discovery and design of small-molecule inhibitors of Bcl-2 and Bcl-xL is a new and exciting research area for development of an entirely new class of anti-cancer drugs to overcome apoptosis-resistance of cancer cells. We will present our latest progress on the discovery and development of novel small-molecule inhibitors of Bcl-2/Bcl-xL as a new class of anticancer agents.

Keywords: apoptosis, novel small-molecule inhibitors, Bcl-2 and Bcl-xL proteins

030 Potent Indole-3-Carbinol-Derived Antitumor Agent with Pleiotropic Effects on Multiple Signaling Pathways

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During the course of tumor progression, cancer cells constitutively up-regulate cell proliferation- and cell survival-regulatory signaling mechanisms, thereby overcoming genomic instability and/or acquiring a drug-resistant phenotype. From a clinical perspective, it is desirable to concomitantly target these molecular abnormalities by using a combination therapy or an agent with pleiotropic effects to optimize therapeutic outcomes. This rationale constitutes the molecular basis for structurally optimizing indole-3-carbinol to develop potent antitumor agents with pleiotropic modes of mechanism. This effort has culminated in the generation of OSU-A9, an acid-stable derivative, which exhibits a two-orders-of-magnitude higher antiproliferative activity than indole-3-carbinol. From a translational perspective, OSU-A9 provides considerable therapeutic advantages over indole-3-carbinol with respect to chemical stability and anti-tumor potency. Relative to indole-3-carbinol, OSU-A9 displayed striking similarities in its effects on modulating the functional status of a multitude of signaling targets relevant to survival and cell cycle progression in cancer cells. These targets include Akt and its downstream effectors GSK3b and Bad, the MAP kinases p38, and JNK, the Bcl-2 family members Bax, Bcl-2, Bcl-xL, and Mcl-1, the inhibitor of apoptosis protein (IAP) survivin, NF-kB, cyclin D1, and the CDK inhibitors p21 and p27. Equally important, OSU-A9 perturbs estrogen receptor signaling, and suppresses HER2 and CXCR-4 expression in breast cancer cells, which has significant implications in the therapy of metastatic, estrogen-dependent cancers. Despite this broad range of antitumor activities, normal prostate epithelial cells and nonmalignant MCF-10A breast epithelial cells were less susceptible to the antiproliferative effect of OSU-A9 than the respective cancer cell lines, reflecting the *in vivo* tolerance of this drug in tumor-bearing nude mice. Our data indicate that oral OSU-A9 at 25 mg/kg/day could effectively suppress tumor xenograft growth in various cancer animal models, including those of prostate, breast, liver, and pancreas, without incurring overt toxicity. The effects of OSU-A9 on various intratumoral biomarkers were qualitatively similar to those observed *in vitro*, indicative of the oral bioavailability and *in vivo* efficacy of OSU-A9 in tumor-bearing mice. Although indole-3-carbinol has been reported to cause centrilobular hepatocellular hypertrophy and to induce hepatic Phase I and Phase II enzymes in rodents, oral OSU-A9 is not a significant inducer of the biotransformation enzymatic system.

Reference: Weng et al. Cancer Res. (2007) 67, 7815-24.

Keywords: indole-3-carbinol, OSU-A9, pleiotropic antitumor agents

031 A Phase II Study of Neoadjuvant Bevacizumab and Radiation Therapy for Resectable Soft Tissue Sarcomas

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Background: Human cancers such as soft tissue sarcomas (STS) often overexpress vascular endothelial growth factor (VEGF), leading to irregular and porous tumor blood vessels. Numerous pre-clinical studies have shown that anti-VEGF agents increase the efficacy of radiation therapy against solid tumors, but there are few clinical trials. Bevacizumab is a humanized anti-VEGF monoclonal antibody that binds VEGF and inhibits its activity.

Methods: We designed a phase II clinical trial to examine the use of neoadjuvant bevacizumab combined with radiation therapy for patients with intermediate- or high- grade, >5 cm STS. Patients received bevacizumab for 2 weeks followed by a combination of bevacizumab and radiation for 6 weeks. Tumors were then resected 6-7 weeks after the completion of therapy. Perfusion CT scans were be used to assess tumor blood flow and vascular permeability during treatment. Serial blood samples were collected to assess changes in circulating angiogenic factors, and serial tumor biopsies were analyzed by gene expression microarray and immunohistochemistry.

Results: Neoadjuvant bevacizumab and radiation resulted in 5 of 12 tumors (42%) with >85% necrosis, 2 tumors (21%) with 50-85% necrosis, and 5 tumors (42%) with <50% necrosis. Historically, radiation alone results in >85% necrosis in only 10-20% of sarcomas. Using perfusion CT parameters, we found that tumors with >85% necrosis following treatment had a dramatic reduction in permeability surface area after just 2 weeks of bevacizumab compared to that had <85% necrosis (18.4 +/- s.d. 17.45 vs. 10.5 +/- 6.5, $p<0.05$). Circulating levels of VEGF and placental growth factor (PlGF) decreased during treatment. Microvessel density per high powered field (h.p.f) decreased from 21.7 +/- 16.6 to 8.8 +/- 6.5 ($p<0.05$) after bevacizumab alone and remained stable following combination therapy (7.4 +/- 6.7). Tumor cell proliferation as measured by PCNA positive nuclei per h.p.f. decreased from 172.9 +/- 109.8 to 85.7 +/- 91.3 ($p<0.05$), and apoptosis as measured by TUNEL positive nuclei per h.p.f. increased from 5.4 +/- 10.6 to 24.8 +/- 21.2) following combination therapy. DNA microarray data are currently being analyzed.

Conclusions: Bevacizumab may increase the efficacy of radiation therapy against STS, and this may translate into a reduction of local recurrences. Sarcomas display wide heterogeneity in their vasculature as measured by several correlative science studies, and these studies may provide biomarkers as to which tumors will respond best to bevacizumab and radiation.

Keywords: radiation therapy, angiogenesis, sarcomas

032 DDX3 Associates With DR5 and Negatively Regulates Apoptosis Signal Transduction of the Death Domain

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Therapeutic agents that target death receptors on tumor cells, including the native ligand TRAIL, and agonistic monoclonal antibodies, have been in the clinical development phase. While these agents are shown to benefit some cancer patients, the resistance of tumor cells to TRAIL-mediated apoptosis has been a major concern. Therefore, understanding of the molecular mechanisms by which tumor cells develop apoptosis resistance to death receptors is important in the development of an effective anti-death receptor strategy for cancer therapy. We have previously reported that tumor cells can develop an inducible resistance to DR5-mediated apoptosis via an as yet unknown mechanism. We have identified DDX3, a member of the DEAD Box protein family, as a novel death receptor-associated adapter protein, which associates with a non-death domain region of the cytoplasmic tail of DR5 and recruits cIAP1 via its Caspase Recruiting Domain (CARD), thereby forming an inhibitory complex of DR5/DDX3/cIAP1 to antagonize the death domain function. A mutant form of DR5 lacking the DDX3 binding domain was more pro-apoptotic than the wild-type form. Knockdown of DDDX3 significantly enhanced DR5-mediated apoptosis. A dominant mutant form of DDX3 lacking the CARD reversed apoptosis resistance in tumor cells. The levels of cIAP1 and other apoptosis inhibitory proteins in the DR5/DDX3 complex were correlated with the susceptibility/resistance of tumor cells to DR5-mediated apoptosis. Thus, the DR5/DDX3/IAP complex might serve as a drug target for specific enhancement of DR5-mediated apoptosis, and might be a critical biomarker to predict tumor cell response to DR5-mediated apoptosis.

Keywords: DDX3, DR5, apoptosis

033 RNAi Analysis and Screening for the Study of Cancer Gene Function and Anti-Cancer Therapeutics

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The identification of the RNAi mechanism in mammalian cells has had a profound impact on our understanding of the post-transcriptional regulation of gene expression. Further, the discovery of RNAi has provided the scientific community with a powerful means for conducting functional studies in a gene-specific manner. Currently, RNAi-based technologies offer the most versatile approach for conducting gene specific loss of function (LOF) studies that are critical for determining normal and/or cancer-related roles of proteins. RNAi-based technologies have the potential to enhance many aspects of the anti-cancer drug development process. For example, we are using RNAi analysis and screening to probe the impact of specific genes on drug-activity, including the study of genes involved in multiple drug resistance and the relationship between *ASNS* gene expression and sensitivity to the anti-leukemia agent L-Asparaginase. We are also using a chemosensitization (synthetic lethal) approach that combines the decrease of a protein through RNAi with administration of a small molecule or biologic to identify new proteins that directly or indirectly modulate the pharmacology of anti-cancer therapeutics. This RNAi screening approach has the potential to enhance the clinical application of an established or investigational drug by, (1) identifying synergistic molecular targets that exploit complementary vulnerabilities within a cancer cell, (2) enabling the use of lower concentrations of a drug that exhibits dose-dependent non-specific toxicities, (3) overcoming drug resistance, and (4) broadening the clinical application of a drug to other cancer types. This approach can also be used to identify antagonists of drug activity that may be relevant to the response of patients to drugs. To date our chemosensitization RNAi screens have led us to investigate the role of the ribonucleotide reductase complex in the cellular response to the topoisomerase 1 inhibitor camptothecin (CPT). We have also identified *MAP3K7* (TAK1) as a potent agonist of CPT and its clinical derivatives. These studies illustrate the importance of RNAi analysis and screening in translational research with a goal of directly impacting therapeutic approaches to cancer.

Keywords: RNAi, chemosensitization, screening

034 Exploiting Gene Expression Profiling to Identify Novel Minimal Residual Disease Markers of Neuroblastoma

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Despite achieving clinical remission, many patients with metastatic neuroblastoma (NB) relapse and die, due in part to the presence of subclinical minimal residual disease (MRD). Immunotherapy and biologic therapy directed against MRD can improve outcome. The ability to measure MRD is critical for gauging the success of these targeting approaches, especially in the key metastatic compartments of marrow and blood. However, no single MRD marker will be adequate because of tumor heterogeneity. Genome-wide expression profiling can uncover novel genes differentially expressed in NB tumors over normal marrow/blood with potential as MRD markers.

Gene expression array was carried out on 48 stage 4 NB tumors and 9 remission marrows using the Affymetrix U-95 gene chip. 34 genes with a tumor-to-marrow expression ratio higher than tyrosine hydroxylase, the most widely used NB marker, were identified. Quantitative RT-PCR was performed on all 34 genes to study the sensitivity range of tumor cell detection and the expression of these genes in normal marrow/blood samples and in stage 4 NB tumors. Top ranking markers were then tested for prognostic significance in the marrows of stage 4 patients collected from the same treatment protocol after 2 cycles of immunotherapy (n=116).

Based on sensitivity assays, 8 top-ranking markers were identified: CCND1, CRMP1, DDC, GABRB3, ISL1, KIF1A, PHOX2B, and TACC2. They were also highly expressed in stage 4 NB tumors (n=20) and had low to no detection in normal marrow/blood samples (n=20). Moreover, expression of CCND1, DDC, GABRB3, ISL1, KIF1A, and PHOX2B in marrows sampled after 2 treatment cycles had prognostic impact on the progression-free and overall survival of these stage 4 patients with a median followup of 5.9 years ($p<0.001$).

In conclusion, marker discovery based on differential gene expression profiling, stringent sensitivity and specificity assays, and well-annotated patient samples, can rapidly prioritize and identify novel MRD markers of neuroblastoma.

Keywords: minimal residual disease, molecular markers, gene expression profiling

035 Understanding and Targeting Cancer Stem Cells

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As we identify and isolate normal tissue-specific stem cells and Leukemia Stem Cells (LSCs), we are assessing the activity of and role of critical molecular signaling pathways in normal stem-progenitor cells and LSCs. For example, our Program Project Grant studies include the following directions:

- Separate LSCs using properties that distinguish normal tissue-specific stem cells from their differentiated progeny. In addition, we will investigate whether the divergent outcomes between "pediatric-type" and "adult-type" acute lymphoblastic leukemia (ALL) are the result of different stem cell populations. In clinical trials, we will test whether targeting ALL stem cells via inhibition of the hedgehog pathway (e.g., Smothened) or telomerase will improve the outcome in adult ALL.
- Determine if the FLT3 mutation is expressed in LSCs from patients with FLT3 mutant acute myeloid leukemias (AMLs). In addition, we will evaluate whether LSCs from patients with FLT3 mutant AML are heterogeneous with respect to response to in vivo treatment with FLT3 inhibitors. We will also study how other oncogenes (known to occur in associate with mutated FLT3 in human leukemia cases) cooperate with mutant FLT3 to transform normal hematopoietic stem-progenitor cells into LSCs. The understanding gained may influence design of future clinical trials employing FLT3 inhibitors.
- Quantify microRNA expression at defined steps of normal human and mouse hematopoietic development, and in LSCs versus the bulk populations of leukemia cells in a given patient sample. In addition, we will determine the cell and molecular mechanisms by which selected microRNAs affect the differentiation and biology of primary human hematopoietic stem-progenitor cells and LSCs. This understanding may inspire clinical trials of microRNA mimics or microRNA antagonists, or of drugs directed at pathways regulated by microRNAs.

Keywords: cancer stem cell; tyrosine kinase; microRNA

036 The Quiescent Cancer Stem Cell and Defective Quorum Sensing: Major Obstacles to Curative Treatment of Cancer

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There is increasing evidence that most lethal cancers are initiated by oncogenic mutations in adult stem cells or in progenitors with innate potential stem cell properties which, when mutated, are able to function as de facto cancer stem cells. Requisite stem cell properties include extensive self-renewing ability, relative resistance to apoptosis and senescence, and the necessity of spending a substantial part of their life-span in a dormancy state to lessen the risk of errors from repeated divisions, and to allow time for repair and elimination of noxious chemicals and toxins, including cytotoxic drugs. Quiescent cancer stem cells are not killed by drugs designed to kill proliferating cells, and their survival is a major reason for failure to cure even highly chemo-sensitive tumors. Another hallmark of cancer stem cells is their failure to respond to normal regulatory mechanisms that curtail cell production when a normal homeostatic cell density equilibrium is reached, balancing cell production and cell death. While it is known that many cell cycle and apoptotic genes and signaling pathways controlling proliferation are dysregulated in cancer cells, little is known about the specific molecular abnormalities responsible for their inability to maintain normal homeostatic cell densities that might be targetable, nor how bacterial quorum sensing (QS) systems regulating cell densities may resemble mammalian QS systems. We have previously found that quiescent CML stem and progenitor cells are more easily triggered into cycle by cytokines than comparable normal cells, suggesting they may be at a later stage of development and less responsive to normal regulatory mechanisms.

The central focus of our research is to search for selective targetable differences between normal and CML quiescent stem cells. We chose CML because highly effective bcr-abl inhibitors are available that can eliminate the great majority of proliferating cells in early stage disease with relatively little toxicity. However, these drugs are not curative and we propose a major reason is because the quiescent CML stem cells are resistant to these drugs as well as to most other drugs yet tested by ourselves and others. Using a wide variety of methods, we have isolated highly purified subpopulations of proliferating and quiescent normal and CML stem and progenitor cells. In quiescent ($CD34+/G_O$) normal but not CML cells compared to cycling cells ($CD34+/G_1SG_2M$) there is down-regulation of CD38 and up-regulation of stem cell-associated genes including ALDH, HLF, and GATA3. In comparing CML and normal $CD34+/G_O$ cells, a number of stem cell associated genes are down-regulated in CML G_O cells, including CD133 (↓20 fold),. MSI2, HLF, GBP2, and DLG7, and there is up-regulation of genes characteristic of erythro-megakaryocyte development (CD36, KLF-Hemoglobin β, δ, γ, TFR2 and CD41, providing additional evidence that the majority of G_O CML cells belong to a more differentiated compartment than the normal G_O cells. Of all the genes up-regulated in CML G_O cells we first focused on the Leptin receptor (LepR) because the Ra isoform is one of the most over-expressed genes (20 fold), and, being a receptor, it might be used to distinguish quiescent CML cells from normal ones and possibly serve as a target for a drug or armed antibody. We are currently further enriching and characterizing normal and CML stem cells (i.e. CD34+CD38-Thy1+CD45RA-) and also examining other differences in signaling molecules and pathways (e.g. CD133, Akt-FOXO, PI3K, lipid raft formation) that we suspect may explain why CML stem cells continue cycling and producing cells long beyond normal homeostatic limits. If we are successful in identifying targetable differences and developing effective selective drugs for quiescent CML stem/progenitor cells, it is likely this research will be applicable to many other types of cancer.

Keywords: cancer stem cell, CML, microarray

037 Targeting Epigenetic Modifications in Breast Cancer

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Purpose: To develop an Early Therapeutics Program with emphasis in Phase II clinical trials at an NCI designated Comprehensive Cancer Center that can support itself as a single institution, thus limiting multi-institution consortia related regulatory, implementation and monitoring issues. In addition to cost savings, this approach would enable interdisciplinary and interdepartmental at a major academic university.

Methods: Clinical trialists and basic scientist with interest in Experimental Therapeutics were assembled into a matrix which incorporated 4 platforms: 1- clinical, 2- correlative science, 3- imaging and radiation, and 4- biostatistics/informatics. An advisory committee and a Data Safety Monitoring Board were established with the purpose of advising and overseeing the activities of this program and his Principal investigator. The clinical platform was composed by A- disease specific groups directed by specific leaders in 1- lung, 2- breast, 3- gastrointestinal, 4- GYN, 5- lymphoid, and 6- myeloid malignancies; B- special populations program with leaders in 1- minority populations, 2-thyroid/neuroendocrine and 3- CNS malignancies; and C- nursing, reporting and regulatory. The Correlative Science platform was composed by groups specializing in assay development & pharmacokinetics, pharmacogenomics, tissue procurement, and biological fluid procurement and processing. Imaging and Radiation was composed by groups specializing in research imaging, radiation oncology and procedural radiology, with a pathologist interfacing with the correlative science platform. Complementing the above was the Biostatistics and Informatics component helping with the design, and data assembly and interpretation.

Results: At 2 ½ years post-implementation (June 30, 2008) this contract has opened 18 clinical trials in 13 different malignancies (hepatocellular, biliary, multiple myeloma, merkle cell, thyroid, melanoma, AML, CLL, myelofibrosis, gliomas, breast, skin and prostate) with 227 patients enrolled to date. Notable examples in relatively hard to accrue malignancies are discussed below:

- GW572016 is a potent and selective dual inhibitor of EGFR and ErbB2 TK activity. We hypothesized that GW572016 should be active in the treatment of hepatobiliary carcinomas given the beneficial clinical effects observed with EGFR-TK inhibition in this group of patients plus the additional blockade of ErbB2 with which EGFR heterodimerizes to transduce signal. Response to or resistance may be controlled by functional expression of the intended target. 28 pts with HCC were accrued. Although no objective responses were observed, 8 (31%) patients had stable disease (SD) including 2 (7.6%) with SD lasting > 6 months. Tissue and blood specimens were available on >95% of patients to evaluate the correlative hypothesis.
- The hypomethylating agent decitabine was evaluated as a single agent in patiets with AML >60 years old, previously untreated, not candidates for or who refused standard induction. The dose was based on a previous NCI sponsored phase I study (NCI 6236, Blum JCO 2007) conducted under the OSU U01, derived from a plan to pick a dose of decitabine based on a gene re-expression endpoint in that study. Induction was for 10 days at 20mg/m^2/day. The post-induction therapy was abbreviated and individually customized to each patient's counts (3-5 days of decitabine/cycle). Cycles were repeated every 4-5 weeks. From May 07 to June 08, 32 pts have been accrued (of a planned 33). Response data on the first 16 patients enrolled 8 achieved CR (by IWG criteria with complete count recovery), 2 went on to alloPB stem-cell transplant, 4 achieved CRi , 4 had progressive disease. Several patients have been in CR 6+ months.

Conclusions: As it has been demonstrated for Phase I trials (U01 mechanism), our experience suggests that contracts for Phase II trials can be successfully allocated to single institutions within the frame of a large academic institution with an NCI designated Comprehensive Cancer Center, at a fraction of the cost required by multi-institutional consortia.

Keywords: clinical trials, early therapeutics, phase II

038 Use of International HapMap Cell Lines for Pharmacogenomic Discovery

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The International HapMap Project provides a resource of genotypic data in human lymphoblastoid cell lines (LCLs) that can be used to identify genes affecting complex traits including gene expression and sensitivity to drugs. We evaluated gene expression and sensitivity to chemotherapy in the full set of HapMap lymphoblastoid cell lines derived from individuals of European (CEU), African (YRI) and Asian (ASN) ancestry. Gene expression was evaluated for 9,156 genes using the Affymetrix GeneChip® Human Exon 1.0 ST Array for CEU and YRI samples. Differences in sensitivity to chemotherapy that could be due to differences in gene expression or in genetic variants affecting protein function were observed between CEU, YRI and ASN cell lines. We therefore developed an unbiased, whole genome cell based model integrating genotype, gene expression and sensitivity of HapMap cell lines to chemotherapeutic drugs. Cell growth inhibition at increasing concentrations of chemotherapeutic agents was evaluated. SNP genotype and the IC_{50} (concentration required to inhibit 50% cell growth) of each agent were linked through whole genome association. A second QTDT association test was performed between SNP genotype and gene expression, and linear regression was then utilized to evaluate the correlation between gene expression and drug IC_{50}. We identified pharmacogenetic signatures significantly associated with sensitivity to cisplatin, carboplatin, daunorubicin, etoposide, capecitabine, araC and pemetrexed and in some instances these genetic variants were associated with gene expression. A multivariate model indicated that a limited number of genetic variants explained approximately 20-65% of the observed human variation in cellular sensitivity to these chemotherapeutic agents. Thus, cell lines derived from CEU, YRI and ASN populations were utilized to build predictive models to identify genetic signatures for susceptibility to chemotherapy-induced cytotoxicity. This unbiased genome-wide approach allowed identification of unique, population-specific pharmacogenomic signatures to explain variation in cellular susceptibility to chemotherapeutic-induced cytotoxicity that may be considered for clinical validation. Furthermore, there are many cellular phenotypes that can be studied in these cell lines to gain insight as to how genetic variation contributes to phenotypic variation. This model can be applied to the identification of genetic variants acting through expression on many cellular phenotypes.

Keywords: capecitabine, pharmacogenetic, genetic association

039 An Orally Bioavailable Small Molecule Inhibitor of Hedgehog Signaling Inhibits Tumor Initiation and Metastasis in Pancreatic Cancer

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Background and Aims: Recent evidence suggests, that blockade of aberrant Hedgehog (Hh) signaling can be exploited as a therapeutic strategy for pancreatic cancer. Our previous studies using the prototype Hh small molecule antagonist cyclopamine had demonstrated the striking inhibition of systemic metastases upon Hh blockade in spontaneously metastatic orthotopic xenograft models. Cyclopamine is a natural compound with suboptimal pharmacokinetics, which impedes the clinical translation of this promising line of therapy. In the present study, a novel, orally bioavailable small molecule Hh inhibitor, IPI-269609, was tested using *in vitro* and *in vivo* model systems.

Methods and Results: *In vitro* treatment of pancreatic cancer cell lines to IPI-269609 resembled effects observed using cyclopamine, namely Gli-responsive reporter knockdown, downregulation of the Hedgehog target genes *Gli1* and *Ptch,* as well as abrogation of cell migration and colony formation in soft agar. Single-agent IPI-269609 profoundly inhibited systemic metastases in orthotopic xenografts established from human pancreatic cancer cell lines, although Hh blockade had minimal impact on primary tumor volume. The only discernible phenotype observed within the treated primary tumor was a significant reduction in the population of aldehyde dehdrogenase (ALDH)-bright cells, which we have previously identified as a clonogenic tumor-initiating population in pancreatic cancer. Selective *ex vivo* depletion of ALDH-bright cells with IPI-269609 was accompanied by significant reduction in tumor engraftment rates in athymic mice.

Conclusions: Pharmacological blockade of aberrant Hh signaling might prove to be an effective therapeutic strategy for inhibition of systemic metastases in pancreatic cancer, likely through targeting subsets of cancer cells with tumor-initiating ("cancer stem cell") properties.

Keywords: pancreatic cancer, metastases, hedgehog

040 Defining Genetic Subgroups of Melanoma and Determining Optimal Targeted Therapy: Parallel Preclinical Studies and Clinical Trials

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The identification of somatic genetic changes that activate signal transduction pathways at distinct points has allowed for the delineation of genetic subsets of tumors for which specific targeted therapies can be developed. Based on mutual exclusitivity of activating mutations in the MAPK pathway, melanomas can be stratified into three groups: *BRAF* (60%), *NRAS* (15%) and *KIT* (3%). However, additional mutations are known to occur in the PI3K and p53/Rb pathways which will likely influence responsiveness to MAPK pathway targeted therapy. We currently characterize these mutations prospectively for patients with metastatic melanoma for inclusion into clinical trials. We are evaluating the first selective BRAF inhibitor (PLX4032) with preclinical activity only in *BRAF*-V600E mutated tumors in a phase I/II trial; a less selective BRAF inhibitor (RAF-265) which demonstrates activity against *BRAF* and *NRAS* mutant cell lines in a separate phase I/II trial; and the c-kit inhibitor, imatinib, in a phase II trial for patients with *KIT* mutations.

While conducting the clinical trials with PLX4032 and RAF-265 we are interested in identifying determinants of therapeutic resistance *in vitro* to BRAF-inhibitors in melanomas that carry the common BRAF V600E mutation. We identified a set of metastatic melanomas and melanoma cell lines, which harbored either concurrent *BRAF* V600E and*CDK4* mutations or concurrent *BRAF* V600E and cyclin D1 (*CCND1*) amplification. We found that cell lines with a *CDK4* mutation alone did not have increased resistance to a BRAF-inhibitor, whereas a cell line with a *CDK* mutation and *CCND1* amplification did. We overexpressed CCND1 alone and in the presence of CDK4 in a drug sensitive melanoma line. CCND1 overexpression alone increased resistance, which was enhanced with concurrent overexpressed of CDK4. Increased levels of cyclin D1, resulting from genomic amplification, may contribute to the BRAF-inhibitor resistance of *BRAF*-V600E mutated melanomas. Secondly, we were interested in determining whether non-V600E *BRAF* mutated melanomas responded differently to therapies than V600E melanomas. We identified two additional subgroups with genetic activation of the MAPK pathway for which distinct therapeutic strategies appear appropriate. Melanoma lines with non-V600E mutations in BRAF (G469E, D549V) are highly resistant to MEK inhibition; however they were sensitive to the CRAF inhibitor sorafenib; unlike *BRAF* V600E cell lines.

Prior studies have shown low-activity mutants of *BRAF* signal via CRAF. Sorafenib down-regulated CRAF targets, suggesting that sorafenib may be ideally suited for this small subset of melanomas. Lastly, we wanted to study melanomas without activating mutations in *BRAF* or *NRAS*, as these constitute a substantial group of tumors. We used a genomic strategy to identify a group of melanomas and melanoma cell lines with co-amplification of *CDK4* and *KIT*. Pharmacological studies showed they were resistant to BRAF inhibitors but sensitive to imatinib in both *in vitro* and *in vivo* melanoma models. This may be a sub-population, in addition to those with KIT mutations, for which KIT inhibitors could be used. Based on our studies, we can suggest optimal therapies for the two novel sub-groups of melanomas, sorafenib (or other CRAF inhibitors) in non-V600E BRAF mutated and imatinib (or other KIT inibitors) *KIT*/*CDK4* co-amplified melanomas. In addition, we have demonstrated in patients with *BRAF* V600E mutations, additional genotyping will need to be done in order to identify factors that confer resistance to Raf inhibitors. In particular concurrent *CCND1* amplification appear likely to confer resistance to BRAF-inhibitors. If validated, subsequent phase II trials would focus on patients whose melanoma harbor *BRAF* V600E, but not *CCND1* amplification. These studies strongly support the genetic classification of melanomas should be done prior to treatment and demonstrate how they can be used to guide therapeutic selection.

Keywords: melanoma, somatic mutations, kinase inhibitors

041 DNA Methylation, Gene Expression, and Genetic Background as Markers for Advanced Melanoma and Responsiveness to 5-Aza-2'-Deoxy-Cytidine Induced Growth Arrest

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Aberrant changes in gene activity due to chromatin remodeling involve methylation/demethylation of cytosine at cytosine-guanine (CpG) pair rich islands in promoter regions and post-transcriptional modifications (acetylation/methylation) of histones. Aberrant gain or loss of DNA methylation causes altered expression of genes associated with the malignant phenotype and can be used as a tumor marker. Furthermore, the reversible nature of epigenetic changes in chromatin is the rationale for clinical trials with DNA demethylation agents such as 5-Aza-2'-deoxy-cytidine (5-Aza-CdR, also known as decitabine), or its analogue 5-azacytidine. However, patients' responses are variable and there is a need for molecular markers that can predict and/or monitor the efficacy of therapy. There is also a need to identify possible targets that can synergize with 5-Aza-CdR, especially since combination therapies have shown to enhance 5-Aza-CdR's anti-cancer effect.

Our goal is to identify genetic and epigenetic markers, as well as gene expression signatures associated with the development of melanoma, resistance and sensitivity to 5-Aza-CdR, and the identification of agents that can act in synergy with the drug. Toward these goals, we monitored the sensitivity of 8 patient-derived tumor cells, most from short-term cultures, to 5-Aza-CdR employing cell proliferation and apoptotic assays. The melanoma cells were subjected to global differential gene expression analyses in response to low concentration (0.2 μM) of 5-Aza-CdR employing NimbleGen whole genome expression arrays. We also performed MeDIP (Methylated DNA Immuno-Precipitation) assays to capture and contrast whole genome expression and methylation status in melanoma and normal melanocytes. Further, we sequenced melanoma cells for known melanoma mutations.

In agreement with the clinical experience, our examination revealed that only a subset of 5-Aza-CdR treated cells exhibited signs of growth arrest and apoptosis. Whole-genome differential gene expression, as well as selective protein analyses, ruled out the involvement of genes and proteins involved in the DNA damage response and p53 induction. The finding is consistent with the fact that we used a low concentration (0.2 μM) of 5-Aza--CdR, which acts by incorporating into DNA and downregulation of DNMT. On a global scale, gene enrichment analysis using Gene Ontology (GO) revealed that 5-Aza-CdR caused differential gene expression of genes associated with the extracellular region, response to wounding, response to external stimulus, protease inhibitor activity, and genes associated with acute inflammatory and immune responses. Experimentally, we have identified several pathways that can lead to growth arrest in sensitive cells: induction of $p21^{Cip1}$, activation of TGFβ pathway genes, and activation of signaling modulators such as PTPN6 and IGFBP5. Furthermore, analyses based on known genetic mutations ruled out the involvement of BRAF mutation in 5-Aza-CdR responsiveness, but underscored the role of activated β-catenin in contributing to 5-Aza-CdR resistance. Based on proteasomal degradation of key proteins, we demonstrated beneficial synergy between 5-Aza-CdR and the inhibitors MG132 and Velcade in 5-Aza-CdR resistant melanoma cell lines. MeDIP analyses followed by bisulfite-modified DNA sequencing revealed a set of genes that are suppressed by promoter methylation in advanced, but not primary melanoma cells, that are slightly re-activated by 5-Aza-CdR. We hypothesized that the components analyzed here could be used as biomarkers for advanced melanoma, patient's selection, monitoring treatment, and devising better synergistic therapy to this DNA modifying agent.

Keywords: MeDIP, growth-arrest pathways, proteasomal degradation

042 Small Molecule Repression of c-Myc Gene Expression in Validated Tumor Types by Targeting Secondary DNA Structures

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c-Myc is overexpressed in the majority of tumor types, but there is not as yet a successful approach to inhibition of its expression that has been translated into the clinic setting. Recent in vitro and in vivo studies in lymphoma (Shachaf et al., 2008, *Cancer Res.,* **68**, 5132–5142), osteosarcoma, and colon cancer (Felsher et al. (2008) *Cancer Res.,* **68**, 3081–3086) have revealed that even the transient repression of c-Myc expression results in selective apoptosis in cancer cells, and in one case (lymphoma), merely turning down the expression of c-Myc leads to shrinking of tumor cells to their normal sizes, restoring their ability to die as they are supposed to do.

In recent work from David Levens' lab at NCI (Kouzine et al., 2008, *Nature Struct. Mol. Biol.,* **15**, 146–154) and the Hurley lab at the University of Arizona (submitted for publication), the molecular machine that controls c-Myc transcription has been characterized. Critically, both the on/off switch and the "cruise control" are controlled by a combination of negative superhelicity resulting from transcription, together with specific DNA elements and associated transcriptional factors. Significantly, the DNA sequence encoding the FUSE element (cruise control) and an NHE III_1 element (on/off switch) assume non-duplex DNA structures (melted duplex, G-quadruplexes, i-motifs) that can be targeted by small molecules. Although less well characterized, a number of other cancer-related targets such as VEGF, Hif-1α, PDGF-A, c-Kit, MYB, and KRAS have similar transcriptional targets (Qin and Hurley, 2008, *Biochimie,* in press).

A high-throughput screen using FRET has been established to identify hits for lead optimization of more drug-like molecules that will modulate c-Myc gene expression by stabilizing the silencer element structures. From this program several structurally distinct scaffolds have been identified and are being moved forward through primary and secondary assays.

There is partial clinical validation of this new class of molecular receptors originating from the Hurley/Von Hoff labs. Quarfloxin (CX-3543), a first-in-class drug that targets a secondary DNA structure (G-quadruplex), is now in phase II clinical trials. The lead compound identified in the Hurley lab was successfully transformed through the medicinal chemistry program at Cylene to the clinical drug Quarfloxin. Quarfloxin disrupts the nucleolin/rDNA quadruplex complexes, inhibits the elongation by RNA polymerase I, and exhibits potent antitumor activity in models of cancer. Quarfloxin entered phase I clinical trials in 2005 and then phase II in 2007. A single-agent phase II clinical trial of Quarfloxin is ongoing in neuroendocrine tumors. It is well tolerated in patients with no substantial toxicities.

In summary, we have identified a promising group of new molecular receptors for drug discovery. c-Myc is selected as the first molecular target, where there is a demonstrated critical need for an approach to modulate gene expression. Recent insights into the molecular machine that controls c-Myc gene expression provides a firm basis for this approach. While the first agent that targets this new receptor type is in phase II clinical trials, it does not target c-Myc transcription. Lead molecules that target c-Myc transcription have been identified that are undergoing lead optimization.

Keywords: c-Myc, small molecule, transcription

043 Molecular Targeting the Biologics of Prostate Cancer Stem Cells

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Cancer stem cells (CSC), also referred to as tumor initiating or tumor progenitor cells, represent a small fraction of the total tumor cell burden. The tumor initiating or CSC, are characterized compared to the bulk of the tumor or progeny cells, as having unique cell surface markers, a stem cell-like gene profile, ability to form colonies in soft agar and establishing heterogeneous tumors from very small numbers of cells. CSC are also highly suspected to be relatively resistant to conventional drug and irradiative therapies, and are reasoned to be the residual "seeds" of relapse. We have characterized the CSC population of cells from established prostate cancer cell lines and patient samples. In vitro, the CSC form spheres of self-renewing cells, with a distinct stem-cell genomic signature and CD44+24- phenotype. Three biological aspects have been studied, self-renewal (sphere formation), differentiation into progeny (heterogeneous bulk) tumor cells, and the ability to invade matrigel (in vitro invasiveness).

The CSC spheres undergo differentiation in the presence of serum or in vivo xenografts. The component of serum has been identified and peptides against the receptor block differentiation of the CSC in vitro and also the establishment of primary tumors from CSC in xenograft models. Thus prevention of the differentiation of CSC into progeny tumor may be a provide and approach to mitigating relapse of residual tumor. Several, phytochemical compounds were found to kill prostate CSC, without affecting normal stem cells. The phytochemicals were also shown to block xenograft primary tumors initiated from CSC and the molecular mechanisms have been determined. Finally, CSC were shown to be the principal subpopulation of tumor cells that undergo EMT and invade matrigel, suggesting that they may be unique in aspects which can contribute to metastatic potential. Together, isolation and characterization of CSC allow the investigation of these unique cellular constituents of tumors so that molecular targets specific to their biologics can be studied.

Keywords: stem cells, prostate, differentiation

044 DNA Methylation and Chromatin Modifications: Mechanisms and Applications in Cancer Therapy

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The long-term objective of this translational research program is to advance our basic understanding of the epigenetic regulation of gene expression in cancer cells and to translate the basic discovery of the molecular mechanism into clinical trials in patients with chronic lymphocytic leukemia (CLL). One of the aims is to identify a set of differentially methylated genes in CLL that acts as a CLL DNA methylation signature stratifying risk groups and potentially predicting prognosis and outcome. This study showed that the *death associated protein kinase 1* (*DAPK1*) , a positive mediator of apoptosis, contributes to familial CLL. The majority of the CD19+ selected CLL samples exhibited high level of methylation of DAPK*1* promoter compared to normal CD19+ B cells from healthy volunteers. *DAPK1* expression in CLL cells from 50 unselected patients also showed statistically significant reduction relative to its expression in normal CD19+ cells. Further, from the therapeutics standpoint, treatment with the DNA hypomethylating agent Decitabine resulted in gradual up-regulation of *DAPK*1 expression in Raji cells whereas untreated and methylated cells did not show any detectable expression. Interestingly a family history of CLL incidence (father, 4 sons, and 7 affected individuals in 3 generations) showed mutation in regulatory sequence upstream of DAPK1 promoter in the CLL allele and reduced expression of *DAPK*1 compared to wild type allele. Reduced expression was followed by promoter methylation in the CLL cells of the affected members. These findings provide new insight into the role of DAPK1 in extrinsic and intrinsic pathways of apoptosis and highlight the importance of normal *DAPK*1 expression in B cells. We are currently exploiting these findings therapeutically through agents targeting epigenetic silencing to re-expresss DAPK1 and explore other genes silenced by methylation in CLL that could also be associated with familial disposition to CLL. The receptor-type protein tyrosine phosphatase gene, *PTPROt*, is another gene that is methylated and silenced in CLL. This gene was methylated in a large population (70%) of primary CLL samples (n=90) . We have established that PTPROt is a novel tumor suppressor. Ectopic expression of *PTPROt* in the non-expressing leukemia cells significantly increased sensitivity of these cells to Fludarabine, a drug used in CLL therapy. Increased apoptosis in PTPROt expressing cells is due to reduced Mcl1 expression. We are exploring the possibility that methylation of PTPROt correlates with the high risk CLL population. The substrates of PTPROt are being explored in order to investigate whether the increased phosphorylation of a key substrate(s) due to *PTPROt* suppression plays a role in CLL tumorigenesis. In another study, mass spec analysis showed significant changes in histone H2A in >80% of the LL patients relative to healthy control samples. These changes correspond to a decrease in the abundance of two specific variants of histone H2A . We are currently determining whether the changes in histone H2A variant abundance seen in CLL patients occur at transcriptional or translational levels. The antibodies of these variants are being currently raised with some degree of success that would allow immunolocalization and functional studies that relate to their role in CLL tumorigenesis. Another research focus is aimed at identification of the target genes regulated by BRG1 and hBRM (chromatin remodeling factors/complex)-associated histone methyltrnasferase PRMT5. This study showed that *the suppressor of tumorigenecity 7 (ST7)* is a direct target of PRNT5 in mantle cell lymphoma. We are now exploring to see whether it is also target gene in CLL and whether there is a direct correlation between PRMT5-mediated symmetric methylation of histones at the promoters of target genes and the expression of these genes in CLL cell lines and primary CLL relative to control samples. Finally, we have performed a phase 1 minimum effective pharmacologic dose (MEPD) study of Decitabine followed by a phase II study of this agent . In this study, re-activation of the genes suppressed in CLL (described above) has been examined to correlate tumor regression with re-activation of specific tumor suppressor genes. In addition, we are pursuing alternative mechanisms to target inhibition of methylation in non-dividing cells that include down-regulation of DNMTs by altering the expression of microRNAs that target DNMT mRNAs.

Keywords: chronic lymphocytic leukemia, epigenetic mechanisms, epigenetic therapy

045 Epigenetics and Chemotherapy Resistance in Acute Myelogenous Leukemia

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DNA methylation of CpG islands around gene transcription start sites results in gene silencing and plays a role in leukemia pathophysiology. Its impact in leukemia progression is not fully understood. We performed genome-wide screening for methylated CpG islands and identified 8 genes frequently methylated in leukemia cell lines and in acute myeloid leukemia (AML) patients: NOR1, CDH13, p15, NPM2, OLIG2, PGR, HIN1, and SLC26A4. We assessed the methylation status of these genes and of the repetitive element LINE1 in 30 patients with AML, both at diagnosis and relapse. Abnormal methylation was found in 23%-83% of patients at diagnosis and in 47%-93% at relapse, with CDH13 being the most frequently methylated. We observed concordance in methylation of several genes, confirming the presence of a hypermethylator pathway in AML. DNA methylation levels increased at relapse in 25 of 30 (83%) AML patients. These changes represent much larger epigenetic dysregulation, since methylation microarray analysis of 9008 autosomal genes in 4 patients showed hypermethylation ranging from 5.9% to 13.6 % (median 8.3%) genes at diagnosis and 8.0% to 15.2% (median 10.6%) genes in relapse (P<.0001). These data suggest that DNA methylation is involved in AML progression to chemotherapy resistance and provide a rationale for the use of epigenetic agents in remission maintenance. Towards that goal, we analyzed AML samples from patients treated with the DNA methylation inhibitor decitabine and showed efficient global (LINE1) and gene specific (P15) hypomethylation and gene reactivation shortly after therapy. A clinical trial testing the use of decitabine in remission maintenance in AML has been initiated.

Keywords: DNA methylation, AML, epigenetic

046 A novel, High-Throughput Biochemical Screen of Canonical Wnt Signaling Identifies a Potent and Specific Inhibitor

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We have performed a novel, high-throughput screen to identify small molecule regulators of the canonical Wnt pathway. Inappropriate activation of canonical Wnt signaling is a critical event in the genesis of many common human cancers, yet the mechanisms that Wnt regulates signal transduction under normal and pathological conditions are poorly understood. β-catenin, a transcription factor, is stabilized by activation of the Wnt pathway. In the absence of a Wnt ligand, a destruction complex assembled by axin targets β-catenin for degradation. A Wnt signal inhibits this complex, thus leading to elevated levels of β-catenin that activate gene transcription. We have previously shown that Xenopus egg extracts accurately recapitulate the degradation of β-catenin and developed a mathematical model of Wnt signal transduction. Based on experimental and theoretical considerations, we hypothesize that Wnt mediated axin degradation is critical for pathway activation and a small molecule which blocks axin degradation would potently drive down β-catenin concentrations and prevent signaling.

We developed a high-throughput assay that recapitulates activation of the canonical Wnt pathway by the Wnt co-receptor LRP6 in Xenopus egg extracts. Addition of β-catenin-firefly luciferase and axin-Renilla luciferase fusion proteins to LRP6 activated Xenopus extracts allowed us to screen drug libraries for regulators of β-catenin and axin turnover. Using this forward chemical genetics approach, we screened natural product and bioactive libraries and identified 19 lead compounds. One of these compounds, VU-WS30, blocks induction of secondary axis formation in Xenopus embryos in a concentration-dependent manner (an indication of Wnt pathway inhibition in vivo). In cultured mammalian HEK293 cells, VU-WS30 inhibits Wnt3a-induced expression from a TOPFLASH reporter (EC50 ~55 nM), as well as the endogenous Wnt gene targets, Myc, Dkk1 and Axin2. Furthermore, inhibition of Wnt-mediated gene transcription by VU-WS30 correlates with decreased cytoplasmic β-catenin levels in these cells. In cancer cells, VU-WS30 inhibits β-catenin-driven proliferation of breast (MDA-MB231) and colon (SW480 and SW620) lines at similar concentrations (EC50 ~55 nM) yet is 100-fold less effective towards non-transformed, non-Wnt signaling human diploid fibroblast, suggesting specificity towards the β-catenin-mediated proliferation. VU-WS30 also decreases cytoplasmic β-catenin levels and synergizes with 5-FU to induce apoptosis in these cancer cells. Actin staining reveals an alteration in cellular morphology suggestive of reversal of an epithelial-mesenchymal transition. *In vivo* studies of vulval and cuticle formation in C. elegans and D. melanogaster, respectively, demonstrate that the molecular target of VU-WS30 is conserved among metazoans. Finally, we show that VU-WS30 is able to inhibit axin degradation in Xenopus extracts and in cell culture, suggesting that VU-WS30 downregulates the canonical Wnt pathway by potentiating the function of axin. We predict VU-WS30 will be a valuable pharmacological agent to study Wnt signaling, and a potential agent for the treatment of Wnt-driven cancers. Of clinical relevance, VU-WS30 has been approved by the FDA for a non-related use.

Keywords: Wnt signaling, LRP6, b-catenin

047 Developing More Stable and Potent DNA Methylation Inhibitors

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Silencing of genes critical for normal cellular functions by aberrant methylation of promoter regions is a hallmark of cancer. Epigenetic therapy with DNA methylation inhibitors results in the demethylation and reactivation of these tumor suppressor genes. Decitabine, or 5-aza-2'-deoxycytidine (5-aza-CdR), is an extremely potent inhibitor of DNA methylation and was recently approved by the Food and Drug Administration for the treatment of myelodysplastic syndrome (MDS). However, since 5-aza-CdR is rapidly degraded by hydrolytic cleavage and deamination by cytidine deaminase there is the need for more stable and potent DNA methylation inhibitors. We have characterized a new demethylating agent, Zebularine {1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one}, that possesses a number of properties desirable for a therapeutic agent. Besides being an effective inhibitor of DNA methylation that preferentially targets cancer cells, zebularine is very stable and has a half-life of approximately 44 hours at pH 1.0 and 508 hours at pH 7.0 making oral administration of this drug possible. Indeed, orally administered zebularine causes demethylation and reactivation of a silenced and hypermethylated p16 gene in human bladder tumor cells grown in nude mice. Furthermore, we have shown that long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. In addition to zebularine, we have developed short oligonucleotides containing 5-aza-CdR that are able to inhibit DNA methylation in cancer cells and protect 5-aza-CdR from deamination *in vivo*. Detailed studies with S110, a 5'-AzapG-3' dinucleotide , showed that it works via a mechanism similar to that of 5-aza-CdR with incorporation of its aza-moiety into DNA. This is the first demonstration of the use of short oligonucleotides to provide effective delivery and prevent enzymatic degradation. These approaches from our lab may pave the way for more stable and potent inhibitors of DNA methylation as well as provide means for improving existing therapies.

References: Cheng et al. JNCI 95(5):339-409, 2003; Cancer Cell 6:151-158, 2004; Cheng et al. MCB 24(3):1270-1278, 2004; Yoo et al. Cancer Res. 67(13):6400-6408, 2007; Yoo et al. Cancer Prev. Res. 2008 in press.

Keywords: methylation inhibitor, Zebularine, 5-Aza-2'-Deoxycytidine

048 Alterations In Endothelial Cell Gene Expression In Human Ovarian Cancer

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Therapeutic strategies based on anti-angiogenic approaches are beginning to show great promise in clinical studies. However, full realization of these approaches requires identification of key differences in gene expression between endothelial cells from tumors *versus* their normal counterparts. Here, we examined gene expression differences in purified endothelial cells from 10 invasive epithelial ovarian cancers and 5 normal ovaries using Affymetrix U133 Plus 2.0 microarrays. Over 400 differentially expressed genes were identified in tumor-associated endothelial cells. We selected and validated 23 genes that were overexpressed by 2.9 to 60-fold using real time RT-PCR and/or immunohistochemistry. Among these, the polycomb group protein enhancer of *Zeste homolog 2*(*EZH2*), protein tyrosine kinase 2 (PTK2, also known as Fak), and the Notch ligand *Jagged1* were elevated 2.9, 3.0, and 4.3-fold, respectively, in tumor-associated endothelial cells. Silencing *EZH2, Fak,* or *Jagged1* genes with short interfering RNA (siRNA) blocked endothelial cell migration and tube-formation *in vitro*. The present study demonstrates that tumor and normal endothelium differ at the molecular level, which may have significant implications for the development of anti-angiogenic therapies.

Keywords: angiogenesis, ovarian cancer, novel targets

049 Targeting Cancer Stem Cells

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Although most hematologic malignancies will respond (often dramatically) to treatment, most patients with these diseases eventually relapse and die of their disease. In fact, it is becoming clear that clinical responses to anticancer therapy often do not translate into improved survivals. The cancer stem cells (CSC) concept is an attractive explanation for the paradox of the relatively poor outcome of cancer patients in the face of high initial response rates. Although generally considered a disease of malignant plasma cells (PC), we found that multiple myeloma (MM) arises from a small population of self-renewing CSC that resemble memory B cells. Not only do these clonotypic B cells circulate in most patients, but they are also resistant to most standard anti-MM agents and thus appear to be responsible for most disease relapses. The MM stem cells resemble their normal counterpart quite closely, such that much of their therapeutic resistance appears to result from mechanisms co-opted from normal stem cells. Reed-Sternberg (RS) cells, the hallmark of Hodgkin lymphoma (HL), are the only blood cells other than PC to occasionally express CD138; this suggests that RS cells represent aberrant PC differentiation, and in fact we found that RS cells express a transcriptional profile similar to normal and malignant PC. Accordingly, we found that HL also appears to arise from CSC that resemble memory B cells, and that these clonotypic B cells circulate in most HL patients even in early stage disease. A characteristic shared with normal stem cells, high expression of aldehyde dehydrogenase (ALDH), is a critical component of our ability to detect circulating CSC in MM and HL.

Although targeting CSC has proved elusive, our data suggest they are still susceptible to novel therapeutic strategies, such as immune-based approaches. Clearly, the allogeneic graft-versus-tumor effect can eliminate hematologic malignancy CSC, but is limited by the toxicity of graft-versus-host disease and the availability of matched donors. About one-third of patients, and most of some ethnic groups, will not have a complete match in unrelated registries. Even when a match can be found, a median of 4 months is required to complete searches; thus, some patients succumb to disease while awaiting identification of a suitable HLA-matched donor. The ability to use haploidentical family members would overcome both of these difficulties. Based on the resistance of normal stem cells to cyclophosphamide (CY) via their high expression of ALDH, the major mechanism of CY inactivation, our group developed high-dose CY post-transplant as a means to induce bidirectional tolerance after allogeneic transplantation. High-dose CY after related haploidentical transplantation results in a toxicity profile similar to that seen with matched sibling donors. Our group also found that T cells present in the bone marrow of MM patients kill MM stem cells, and that regulatory T cells play an important role in down-regulating anti-tumor immunity. Several signaling pathways that are important for the generation and maintenance of normal stem cells during embryonic development or postnatally (eg, Hedgehog or telomerase) also are important for the growth of many cancers. Inhibition of these pathways, even when they are not mutated or overexpressed, produces potent antitumor activity across a range of CSC *in vitro*, possibly because of the key roles these pathways play in stem cell maintenance and growth. We found that induction of CSC terminal differentiation also holds therapeutic promise. Clinical trials targeting CSC immunologically and via these stem cell pathways are currently ongoing.

Assessing the effects of new therapies on CSC will require new clinical paradigms and methodologies that evaluate the effects of therapies on the rare CSC, since traditional response criteria measure tumor bulk and may not reflect changes in rare CSC populations. We believe new paradigms should rely heavily on preclinical modeling, utilize non-traditional trial endpoints (other than clinical response), and evaluate novel preclinical assays that assess the fate of CSC. Accordingly, we recently found that quantifying MM stem cells *in vivo* predicts progression-free survival after MM stem cell directed therapy.

References: Matsui *et al.* Cancer Res. 68:190-7, 2008; Luznik *et al.* Biol Blood Marrow Transplant. 14:641-50, 2008; Huff *et al.* Blood. 107:431-34, 2006; Noonan K *et al.* Cancer Res 65:2026-2034, 2005.

Keywords: cancer stem cells, blood and marrow transplantation, multiple myeloma

050 Orthotopic Transplantation of Lung Cancer Stem Cells From Fresh Surgical Specimen as a Clinically Relevant Mouse Models of Non-Small Cell Lung Carcinoma

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Background. There is an urgent need for animal models that will not only replicate the biology of non-small cell lung cancer (NSCLC), but also preserve their cancer stem cell pool. Evidence is accumulating that most, if not all, malignancies are driven by "a cancer stem cell compartment." As our ability to attack specific targets increases, a fundamental question remains, "Are we targeting the cancer stem cell pool"?

Methods and Results. We have recently identified cancer stem cells in (NSCLC) and have made the primary goal of our research to develop novel clinical relevant animal models that can reliably preserve cancer stem cell pools even during serial in vivo sub-transplantations. Using magnetic bead cell sorting we isolated a population of lung cancer cells carrying the CD133+/CD34- phenotype. These cells express the germ cell markers NANOG, SOX2, KLF-4, CD133 and OCT4 and were highly efficient at initiating tumors when injected into immuno-compromised NOD/SCID mice. In a small pilot project, we isolated CSCs from fresh surgical specimens and were able to maintain these cells in culture as tumor spheres for up to 6 moths and even to orthotopically transplant them serially into immuno-compromised NOD/SCID mice. We also found that these CD133+/CD34-cells were inherently more resistant to chemotherapy agents and radiotherapy. Furthermore, we found activation of the IGF pathway in cells carrying the CD133+/CD34- phenotype but not in CD133-/CD34+ cells. Since we have developed the skills and tools for isolating lung cancer CSCs are currently working on a large scale project aimed at establishing xenografts of lung cancer stem cells (CD133+/CD34-) from up to 50 fresh surgical specimens.

Summary. We have been able to isolate, maintain and propagate lung CSCs derived from fresh surgical specimens. We are in the process of demonstrating that the major abnormalities of the original tumors are also present in the transplanted tumors, even after multiple transplantations. Additionally, we are using this *in vitro* and *in vivo* model as a tool for testing for validation and testing of new and established anticancer agents *in vitro* and *in-vivo*.

Keywords: lung cancer, cancer stem cells, drug development

051 Targeted Therapy of Kaposi's Sarcoma-Associated Herpesvirus (KSHV)-Associated Multicentric Castleman's Disease (MCD) Using Antiviral Drugs that are Activated to Toxic Moieties by KSHV Lytic Genes

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MCD is a hematological B-cell hyperproliferative disorder characterized by fatigue, fevers, cytopenias, elevated serum C-reactive protein (CRP), and excessive levels of interleukin-6 (IL-6). Nearly all cases of MCD arising in HIV-infected patients (pts) are caused by KSHV, and in pts with KSHV-MCD, symptoms are largely caused by excessive production of a KSHV-encoded IL-6 (vIL-6). KSHV, also called human herpesvirus-8 (HHV-8), is also the causative agent for Kaposi's sarcoma (KS), and primary B cell lymphoma (PEL). There is no standard therapy for KSHV-MCD, although there are reports of pts responding to a variety of agents including interferon alpha, cytotoxic chemotherapy, or ganciclovir. Overall, prognosis is poor (median survival 14 months). Two lytic KSHV kinases, encoded by ORF21 and ORF36, can phosphorylate zidovudine (AZT) and ganciclovir to toxic triphosphate moieties. We found that when these lytic genes are activated, as by hypoxia, PEL cells can be killed by AZT plus ganciclovir (Cancer Res 2007; 67:7003–10). We are exploring the translation of these observations to the clinic in pts with KSHV-MCD, taking advantage of the expression of lytic KSHV genes in the tumor cells. Ten pts with biopsy-confirmed KSHV-MCD and constitutional symptoms were treated with high dose oral zidovudine (HDAZT), 600mg every 6 hr, and valganciclovir, a pro-drug of ganciclovir, 900mg every 12 hr. The first-cycle treatment length ranged from 7-21 days. The length of subsequent cycles was 21 days with treatment administered during the first 7 days. Treatment was stopped when pts achieved a complete response (CR) or plateau in response. Pt characteristics: median age 40 yr (range 33-56); median ECOG performance status 2 (1-3); median CD4 count 189 cells/μL(range 19-1319); and median HIV viral load <50 copies/ml^3 plasma (range <50 to 27,500). All pts were on combination antiretroviral therapy; this was adjusted during the time pts were on AZT. Eight pts had a history of KS. All had elevated CRP above 0.8mg/dl (median 13.1, range 1.06-38.7 mg/dl) at treatment initiation. A total of 112 cycles have been administered to date, with a median of 10 (range 3-29) cycles per pt. Nine of 10 pts had documented improvement in constitutional symptoms, CRP levels, or cytopenias. The median survival has not been reached; the 12 month probability of survival is 70%, and patients remain alive from 12.5 to 32 months. The median PFS is 5.4 months; two patients have not yet progressed after 15.5 and 27 months on study**.** Seven pts remain alive. Treatment was well tolerated; toxicity included two pts with fatigue (grade [gr] 3), 1 with nausea (gr3), 1 with transaminitis (gr3) and one with insomnia (gr3). Grade 3 or 4 hematologic toxicity not attributable to disease was seen in only 2 pts. Two pts developed KSHV-associated lymphoma. Three infectious events occurred, a staphylococcal skin abscess, streptococcal meningitis and a streptococcal pneumonia. There were no neutropenia-associated infections. These preliminary data suggest that KSHV-MCD may respond to therapy that targets to two unique viral enzymes. Accrual continues. This research was supported by the Intramural Research Program of the NIH, NCI.

Keywords: Kaposi's sarcoma-associated herpesvirus, Castleman, HIV

052 KLF4 Regulates Notch1 Transcription and Signaling During Epithelial Transformation

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The Notch1 and KLF4 transcription factors function in specification of epithelial cell fate. In a context-dependent manner, each can also act as an oncogene or a tumor suppressor. We expressed a conditional allele of KLF4 in rodent RK3E cells, an epithelial model used previously for analysis of Notch1-induced transformation. In these cells KLF4 rapidly induced expression of *Notch1* mRNA and the active, intracellular form of Notch1. In human breast tumors and in the skin of KLF4-transgenic mice, KLF4 and Notch1 protein levels were strongly correlated, consistent with reports that these are markers of an aggressive subset of cancers. Chromatin immunoprecipitation analysis showed that KLF4 binds to the Notch1 promoter in MCF10A mammary epithelial cells, and KLF4 could activate transcription through a minimal Notch1 promoter fragment. siRNA-mediated suppression of KLF4 gave reduced expression of Notch1 in human mammary cancer cell lines, and knockdown of Notch1 by siRNA or by inhibitors of gamma secretase (GSIs) suppressed transformation by KLF4. Unexpectedly, canonical Notch1-CSL signaling was repressed by KLF4. Consistent with a role for a non-canonical Notch1 pathway in KLF4-mediated transformation, dominant-negative inhibitors of canonical Notch1 signaling (CSL, MAML1) did not block KLF4-mediated transformation of RK3E, while they significantly suppressed transformation by Notch1. Thus, Notch1 can transform cells by distinct mechanisms: the canonical pathway in the absence of KLF4, or a non-canonical pathway in its presence. KLF4 regulation of Notch1 in epithelia may contribute to phenotypes previously associated with these proteins, including tumor progression in the skin or breast. Consequently, KLF4 expression as determined by immunostaining may modify the therapeutic response to Notch inhibitors, such as GSIs, that are currently in clinical trials.

Keywords: klf4, notch1, gamma secretase inhibitors

053 Identification of Cells Initiating Human Melanomas

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Tumor initiating cells capable of self-renewal and differentiation, which are responsible for tumor growth, have been identified in human haematological malignancies and solid cancers. If such minority populations are associated with tumor progression in human patients, specific targeting of tumor initiating cells could provide for a novel strategy to eradicate cancers currently resistant to systemic therapy. Here, we identify a subpopulation enriched for human malignant melanoma initiating cells (MMIC) defined by expression of the chemoresistance mediator ABCB5 and show that specific targeting of this tumorigenic minority population abrogates tumor growth. $ABCB5^+$ tumor cells detected in human melanoma patients display a primitive molecular phenotype and correlate with clinical melanoma progression. In serial human to mouse xenotransplantation experiments, $ABCB5^+$ melanoma cells possess greater tumorigenic capacity than $ABCB5^-$ bulk populations and re-establish clinical tumor heterogeneity. In vivo genetic lineage tracking demonstrates a specific capacity of $ABCB5^+$ subpopulations for self-renewal and differentiation, because $ABCB5^+$ cancer cells generate both $ABCB5^+$ and $ABCB5^-$ progeny, whereas $ABCB5^-$ tumor populations give rise, at lower rates, exclusively to $ABCB5^-$ cells. Systemic administration of a monoclonal antibody directed at ABCB5, shown capable of inducing antibody-dependent cell-mediated cytotoxicity in $ABCB5^+$ MMIC, inhibits tumor xenograft formation and growth in a proof-of-principal analysis involving a limited number of clinical melanomas. Identification of tumor initiating cells with enhanced abundance in more advanced disease but striking susceptibility to specific targeting via a defining chemoresistance determinant has important implications for cancer therapy.

Reference: Schatton T et al. Nature. 2008 Jan 17;451(7176):345-9.

Keywords: tumor initiating cells, ABCB5, melanoma

054 Targeting Signaling Networks via RNAi Approaches to Improve the Treatment of Patients With Ovarian Cancer

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Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancy, and the fifth most common cause of cancer death among American women. Treatment of EOC poses formidable challenges, primarily because of the drug resistance ultimately developed by EOC to chemotherapy. Although many trials currently underway focus on protein-targeted therapies, many are designed without reliable preclinical research showing that the therapeutic targets are truly essential for ovarian tumor growth and survival. Moreover, insights from treatment of other cancer types suggest improved treatment requires the use of two or more targeted therapies used in combination. The underlying theoretical basis for this observation is likely that at time of presentation, most cancers are no longer dependent on single genetic determinants for growth and/or survival. Therefore, there is a growing appreciation of the complex interconnection of signaling networks and a need to eliminate multiple components simultaneously to block potential rescue routes.

Our studies are based on the concepts that (1) for targeted therapies to work, a patient's tumor must be "addicted" to the targets, i.e., the activation of targeted proteins and their downstream pathways are essential for tumor maintenance; and (2) efficacy of targeted therapies will be increased by identifying and simultaneously blocking "rescue pathways" that contribute to signaling network robustness proximal to the target. The quandary for EOC is whether there are unique, tumor-specific genes that are subjects of "addiction". Many candidate targets, such as EGFR and SRC, have been and are currently being assessed in EOC; to date, inhibition of few of these has had significant clinical impact. However, the rapidly growing number of targeted therapeutic agents makes it impossible to test all random combinations of two or more targeted drugs. Therefore, we used mid-throughput siRNA screens to identify proteins that modify response to a number of molecular targeted agents such as erlotinib and panitumumab (targeting EGFR), and dasatinib (targeting SRC), that are effective in other cancer types. In designing a library for this screen, we focused on proteins physically and/or functionally linked to genes already implicated in EOC (e.g., EGFR, HER2, K-RAS, Src, PI3K, AKT) and designed a custom siRNA library targeting 638 genes using 1,276 individual siRNAs. From these "synthetic lethality" screens we uncovered components of several key pathways that synergize with EGFR and/or Src-inhibitors, such as STAT3, NFkB, and Aurora-A. Although some of our hits are not surprising as sensitizers (e.g. AKT2, PIK3CA), some of the strongest hits are entirely unexplored in the context of regulating targeted drug response. We view these as particularly valuable in that they suggest completely new directions in which to consider drug sensitization, both at the mechanistic and the clinical level. Overall, these studies support creating a new bioinformatics-based paradigm for the design and implementation of multi-agent combination trials that should improve the survival of patients with EOC.

Keywords: small interfering RNA (siRNA), chemosensitization, tyrosine kinases

055 Nuclear Structure and Cancer

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Our program project is an integrated, multidisciplinary team approach to experimentally address components of nuclear organization that are functionally linked to modified transcriptional control in transformed and tumor cells. Our working hypothesis is that parameters of nuclear structure support cell growth and phenotypic properties of normal and tumor cells by facilitating the organization of chromosomes, chromatin, genes, transcripts and regulatory complexes within the dynamic three-dimensional context of nuclear architecture. In a highly collaborative setting, this Program Project has been instrumental in establishing the contributions of multiple components of nuclear organization to gene regulation. We have advanced understanding of intranuclear trafficking of transcription factors, chromatin remodeling, chromosome segregation, structural and functional properties of the nuclear matrix, and functional nuclear domains. We are further defining mechanisms that mediate nuclear structure-gene expression interrelationships and will relate changes in nuclear morphology to aberrant growth of tumor cells. Emphasis is on impaired subnuclear organization and assembly of gene regulatory machinery in nuclear microenvironments of metastatic breast cancer and leukemia cells that are associated with compromised biological control. We combine cellular, biochemical, genetic, and molecular approaches together with in vivo and in vitro model systems to investigate: (1) Mechanisms by which nuclear structure integrates physiological regulatory signals that converge to support gene expression; (2) chromatin remodeling mechanisms that render promoter elements accessible to transcription factors and coregulatory proteins; (3) centrosome organization in relation to altered assembly of the mitotic apparatus, chromosome segregation, nuclear structure/function, and aneuploidy; (4) mitotic distribution of regulatory complexes to support post-mitotic transcription; (5) perturbed intranuclear trafficking of tissue-specific transcription factors with transformation, leukemogenesis and osteolytic activity of metastatic breast cancer cells *in vivo*.

This program is defining fundamental structural properties of the cell nucleus that support biological control and are disrupted in leukemia and metastatic breast cancer. We are characterizing components of nuclear organization that can be targeted for innovative cancer therapies.

Keywords: epigenetic control, cell proliferation, nuclear microenvironments

056 Targeting Developmental Signaling Pathways, Such As Hedgehog/Smoothened, In Cancer

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Up-regulation of the developmental signaling pathways, such as Hedgehog/Smoothened signaling pathway plays a key role in the pathogenesis of many devastating malignancies, and are often surface expressed, rendering them susceptible to antibodies and small molecule inhibitors. In recognizing the urgent need to develop new therapies we have exploited intracellular trafficking of key receptors to develop assays that identify novel antibodies and compounds with therapeutic potential that block receptor signaling including Hedgehog/Smoothened. We have established a state-of-the-art image based drug and antibody discovery infrastructure based upon our research to systematically search for small molecule compounds and antibodies with anti-cancer properties. Recently, as part of our ongoing screening project, we have identified by primary and secondary screening more than forty potent inhibitors of Hedgehog signaling. We are now performing studies that will provide the biological rationale and preclinical information to help guide the clinical development of Hedgehog/Smoothened inhibitors that have desirable characteristics (e.g., linear pharmacokinetics, poor inducer or inhibitor of P450 enzymes). Our goal is to be able to file an Investigational New Drug Application (IND) for its therapeutic use within three years. Hedgehog/Smoothened antagonists may also be effective in cancers of the pancreas, brain, prostate, lung, certain skin cancers and liver.

Keywords: therapies, small molecules, antibodies

057 Biology and Prognostic Significance of FLT3 Mutations in AML

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FLT3 encodes a receptor tyrosine kinase that regulates hematopoietic stem cell differentiation and proliferation. Activating mutations of FLT3 can occur as a result of an internal tandem duplication of the juxtamembrane domain (FLT3 internal tandem duplication, FLT3/ITD) or a missense mutation of the activation loop domain (FLT3 activation loop mutation, FLT3/ALM). FLT3/ITD occurs in acute myeloid leukemia (AML) with an age-dependent increase in prevalence from <1% in infant AML to >40% in those older than 60 years, and its presence is associated with high relapse risk and poor outcome. Incidence of FLT3/ALM remains constant in (5-7%) in all age categories, and its presence does not have prognostic significance. Although FLT3/ITD portends higher relapse rate, 25-30% of those with FLT3/ITD do not relapse. We have demonstrated that structural characteristics of FLT3/ITD may affect its biology and clinical significance. Allelic variation of FLT3/ITD, which is a measure of mutant to wild type allele, accurately defines relapse risk, where those with FLT3/ITD allelic ratio (ITD-AR) of greater than 0.4 are at extremely high risk of relapse (>90% RR), whereas those with lower allelic ratio have a RR similar to FLT3 wild type (FLT3/WT) patients. Allelic ratio determination of FLT3/ITD as means of risk-status determination has been incorporated into the current phase III COG AML trial, where those with high ITD-AR are allocated to receive allogeneic stem cell transplant in first CR. Underlying mechanism for allelic ratio variation was studied using SNP/CGH array, demonstrating that copy-neutral LOH (CN-LOH) mediates the allelic variation. Expression profiling in those with CN-LOH identified dys-regulation of genes involved in homologous recombination and DNA segregation pathway. Specific genes are under evaluation for their role in mediating CN-LOH, which may be related to disease resistance.

Structural analysis of FLT3/ITD revealed that in all cases of ITD, amino acid residues Y591-Y597 were duplicated. This region, which encodes the switch and zipper regions of the juxtamembrane (JM) domain of FLT3, plays an important role in directing an optimal orientation of the autophosphorylation ‘switch’ residues and maintaining the autoinhibited conformation. JM-ITD insertions are expected to disrupt the autoinhibited conformation of the JM switch (JM-S) region, thus preventing proper kinase inhibition. In addition, length and the region of the mutation impacts clinical outcome, where those with longer ITD or those ITDs that involve the STAT5 binding domain (Y589/591) have a higher incidence of relapse and worse outcome.

Evaluation of FLT3 expression level in patients with FLT3/WT demonstrated significant variation in FLT3 expression where nearly 1/4th of the patients had 20-100 fold higher FLT3 expression compared to the normal marrow controls. High FLT3/WT expression was associated with higher relapse risk. Leukemic blasts with high FLT3/WT expression had a higher response rate to FLT3 inhibitor Lestarutinib, suggesting that patients with high FLT3/WT expression may respond to FLT3 inhibitors. We demonstrate a level of complexity in FLT3 structure and function not previously appreciated. Complete understanding of the underlying mechanism and biology for FLT3 structural and expression variation would enable more appropriate therapeutic intervention.

Keywords: FLT3, Internal tandem duplication, acute myeloid leukemia

058 Normal and Neoplastic Growth in the Brain

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Brain tumors are the most common group of solid malignancies in children, causing devastating mortality and morbidity in a very understudied patient population. The goal of this program project is to improve understanding and treatment of pediatric brain tumors. Project and core leaders work together to identify key signaling pathways underlying the development of pediatric brain tumors, develop novel *in vitro* and *in vivo* models to study these pathways in the brain, and use these models for relevant preclinical testing of new therapeutic agents. With an initial central focus on medulloblastoma and cerebellar growth regulation, we developed novel mouse models for this tumor and identified a common molecular fingerprint associated with dysregulated Sonic hedgehog (Shh) signaling in mouse medulloblastoma, and in a subset of human medulloblastomas. A small molecule inhibitor of Shh signaling eliminated medulloblastoma in mice; however, it also caused defects in bone development in the treated animals. Based on extensive preclinical data, a Phase I trial of a hedgehog inhibitor for children with recurrent or refractory medulloblastoma will be conducted. This clinical trial will include biological studies to validate molecular markers that may be used to stratify patients in future trials. Similar approaches are used in ongoing studies within the program to address a broader range of pediatric brain tumor types including pediatric high-grade glioma, diffuse brainstem glioma, choroid plexus carcinoma, atypical teratoid/rhabdoid tumors, and a continuing in-depth analysis of medulloblastoma. Projects incorporate analyses of human tumors, novel mouse tumor models, tumor stem cells and micro-RNA regulation. Additionally, mechanistic studies of key signaling pathways involving PI3-kinase, SHH, WNT, BMP and DNA damage responses will further illuminate our understanding of brain tumorigenesis. Our integrated analyses will identify common and unique signal transduction pathways in pediatric brain tumorigenesis, and test the efficacy of inhibitors of these pathways in appropriate preclinical models.

Keywords: pediatric brain tumors, mouse models, signal transduction

059 The Role of Amplification in Predicting Clinical Outcome of Patients With Alveolar Rhabdomysarcoma

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Alveolar rhabdomyosarcoma (ARMS) is an aggressive pediatric soft tissue cancer related to the skeletal muscle lineage. The majority of ARMS tumors have a recurrent chromosomal translocation: either a 2;13 or a 1;13 translocation that generates a *PAX3-FKHR* or *PAX7-FKHR* gene fusion. Within the ARMS category, there is clinical and genetic heterogeneity, consistent with the premise that "primary" genetic events collaborate with "secondary" events during ARMS pathogenesis and give rise to subsets with varying clinical features. As evidence of these secondary events, comparative genomic hybridization studies revealed that genomic amplification occurs frequently in ARMS and localized the most common amplicons to the 12q13-q15 and 2p24 chromosomal regions. This study will focus on amplification of these regions in ARMS as potential molecular markers for clinically relevant subsets.

To localize the 2p24 and 12q13-q15 amplicons, we analyzed genome-wide copy number in 57 ARMS cases using Affymetrix GeneChip Human Mapping arrays. For the 2p24 chromosomal region, an amplification event was identified in 5 of 57 cases. The minimum region of amplification in the five cases was localized to a 0.83 Mb region, which contains two genes, *DDX1* and *MYCN*. For the 12q13-q15 region, we identified two distinct amplification events in 9 of the 57 cases, 8 involving the 12q13.3-q14.1 region (henceforth referred to as 12q14) and 3 involving the 12q15 region. We focused on the more common 12q14 amplification event and localized the minimal common amplified region to a 0.55 Mb region, which contains 27 genes, including *CDK4*.

Based on this mapping data, we developed fluorescent *in situ* hybridization assays to measure copy number of the 2p24 and 12q14 amplified regions. In the 2p24 region, amplification was detected in 17 of 126 ARMS cases (16%). This amplification was ~8-fold higher in PAX3-FKHR- and PAX7-FKHR-positive tumors than in fusion-negative tumors (18% and 22% vs. 2.4%, $p = 0.018$). In the 12q13-q14 region, amplification was detected in 13 of 109 ARMS cases (12%). This amplification was significantly higher in PAX3-FKHR-positive than in PAX7-FKHR-positive (24% vs. 4%, $p < 0.05$) and fusion negative cases (24% vs. 0%, $p = 0.0027$).

In outcome analysis, 2p24 amplification was not associated with a significant difference in failure-free ($p = 0.57$) or overall survival ($p = 0.87$). In contrast, there was a statistically significant association of 12q14 amplification in both failure-free and overall survival. In particular, in ARMS tumors with 12q14 amplification, the hazard ratio in failure-free and overall survival was increased to 2.24 ($p = 0.023$) and 2.26 ($p = 0.040$), respectively. Furthermore, despite the frequent occurrence of 12q14 amplification in PAX3-FKHR-positive tumors, which also have a poor prognosis, the outcome difference associated with 12q14 amplification persists when the tumors are controlled for fusion status. However, in a multivariate analysis with known clinical prognostic variables, 12q14 amplification was not yet a significant independent predictor of outcome ($p = 0.19$) with the current number of test cases.

In summary, these studies have identified amplification of the 12q13.3-q14.1 chromosomal region as a novel molecular marker of poor outcome for the pediatric cancer ARMS. Though previous studies of this amplicon in other tumor types have focused on the CDK4 gene in this region, there are 27 genes contained within the minimal common region of amplification in ARMS and thus multiple genes may be contributing to the action of this amplification event.

Keywords: rhabdomyosarcoma, amplification, fluorescence in situ hybridization

060 Genomic Analysis Using High-Density SNP-Based Oligonucleotide Arrays, Sequencing and MLPA Provides a Comprehensive Analysis of INI1/SMARCB1 in Malignant Rhabdoid Tumors

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Malignant rhabdoid tumors (MRT) are rare, highly aggressive neoplasms found most commonly in infants and young children. Although they may present in any location in the body, they are predominantly found in the central nervous system and kidney, and comprise approximately 1–2% of infant brain and renal tumors. The *INI1/hSNF5/SMARCB1/BAF47* gene in 22q11.23 has been implicated in the development of MRT. Previous studies have suggested that approximately 75% of rhabdoid tumors have a detectable mutation/deletion of *INI1*. To date, no other mechanism has been elucidated to explain the remaining 25% of cases. In the present study, the Illumina 550K BeadChip SNP array was used to detect copy number changes and loss of heterozygosity in genomic DNA from 51 rhabdoid tumors (34 brain, 2 spinal cord, 8 renal, 7 extra-renal). Results were analyzed using Beadstudio software provided by Illumina and in-house analysis tools developed to identify copy number alterations. Forty-nine of 51 tumors had detectable aberrations involving 22q11.23, including 11 tumors with heterozygous deletions, 24 tumors with homozygous deletions, and 14 tumors with copy number neutral loss of heterozygosity. The majority of tumors had a variable number of other potential disease associated copy number variations but none were consistently found across multiple patients. Several cases had complex alterations of chromosome 22 in addition to the deletions in the 22q11.2 region. Two of 51 cases did not have detectable abnormalities of 22q11.2 by the array analysis, however interphase FISH and/or MLPA analysis revealed alterations of *INI1* that may have been below the resolution of the array. Twenty-four of the 51 tumors had homozygous deletions of INI1, and 27 tumors had loss of one copy of 22q11.23. Deletions or duplications of one or more exons were identified by MLPA, and coding sequence mutations within individual exons were identified by direct sequence analysis of PCR products. Intragenic deletions/duplications and mutations were identified in 26 of the 27 tumors, consistent with the second inactivating event. These studies suggest that a combination of approaches utilizing high density arrays, FISH, MLPA and *INI1* sequence analysis will detect inactivating deletions and mutations of the *INI1* gene in 98% of primary rhabdoid tumors. Similar approaches have also been used to identify germline deletions and mutations in the *INI1* gene, which predispose carriers to development of rhabdoid tumors. Evaluation of *INI1* status in newly diagnosed patients is recommended to determine eligibility for rhabdoid tumor treatment protocols, as well as for future studies aimed at elucidating the relationship of germline versus somatic *INI1* alterations on clinical outcome.

Keywords: rhabdoid, INI1/SMARCB1, array

061 Del (6)(q22) and BCL6 Rearrangements Detected by Interphase Fluorescence *in-Situ* Hybridization (FISH) are Unfavorable Cytogenetic Abnormalities in Immunocompetent (IC) Patients With Primary Central Nervous System Lymphoma (PCNSL)

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The aim of this study is to determine the prevalence and survival impact of del (6)(q22), *BCL6*, immunoglobulin heavy chain (*IGH*), and *MYC* gene rearrangements in IC PCNSL. We studied 76 HIV-negative IC PCNSL newly diagnosed patients treated between 1992 and 2006. Median age was 63.5 years (range 26-87 years). There were 51 deaths. Median follow-up for 25 alive patients was 399 days (range 0-2520 days). FISH used two-color break apart probes (BAP) for BCL6 and MYC, two-color dual-fusion probes for IGH-BCL6, and two-color probes for del(6)(q22) on thin sections of paraffin-embedded tumor samples. Survival was calculated from the date of tissue diagnosis to date of death or date of last contact using Kaplan-Meier method. The log-rank test was used to compare survival across groups.

31 (41%) cases did not show del (6)(q22) or *BCL6* rearrangement and had a median overall survival (MS) of 731 days (d). 28 (36%) cases showed an isolated del (6)(q22) and had a MS of 412 d (p=0.0048). 17 (23%) cases showed a *BCL6* rearrangement and had a MS of 442 d (p=0.0048). Of the cases with *BCL6* rearrangement, 8 (11%) showed *IGH-BCL6* fusion and the remaining 9 (12%) involved translocation to an unknown gene partner. 2 (3%) cases showed *MYC* translocation to an unknown gene partner. Six cases with *BCL6* rearrangement also showed del (6)(q22). A total of 34 (45%) cases showed del (6)(q22) and had a MS of 412 d (p=0.0198).

In this study, del (6)(q22) and *BCL6* rearrangements were present in 45% and 22% of cases, respectively and were both associated with decreased survival, seemingly independent of patient age and treatment time trends. *IGH* translocations were seen in only 13% of cases, which is less frequent than seen in systemic diffuse large B-cell lymphoma, suggesting a distinct pathogenesis.

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Keywords: PCNSL, tumor suppressor gene, biomarkers

062 Molecular Diagnosis and Target-Directed Therapy in Non-Hodgkin Lymphoma

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Non-Hodgkin Lymphoma (NHL) is very heterogeneous and the different types of NHL vary in their lineage derivation, pathogenesis, clinical behavior and response to treatment. Even within a single diagnostic entity, there is significant heterogeneity in survival indicating divergent activation of oncogenic pathways. We have studied over 2,000 NHL cases to dissect and identify the molecular abnormalities affecting the different types of lymphoma using global approaches such as gene expression profiling (GEP) and array comparative genomic hybridization (aCGH). The goal is to provide an accurate diagnosis for each lymphoma at presentation that will also include key abnormal oncongenic pathways so that therapy may be directed against these pathways.

In the most common type of NHL, the diffuse large B-cell lymphoma (DLBCL), we have identified 3 major subtypes, the germinal center B-cell like (GCB), the activated B-cell Like (ABC), and the primary mediastinal (PM)-DLBCL. They differ in their GE profiles, pattern of genetic abnormalities and clinical outcome. Notably, the NF-$_K$B pathway tends to be highly active in the ABC-DLBCL and we have identified mutations of the coiled-coil domain the *CARD11* gene as one of the mechanisms of NF-$_K$B activation. Other mechanisms may include over-expression of MALT1 associated with 18q21 gain/amplification and possible mutations of other members of this pathway. Interestingly, gain/amplification affecting the microRNA cluster Mir17-92, occurs exclusive in the GBC-DLBCL. Increased expression of Mir17-92 down modulate PTEN and BIM1 expression leading to enhanced AKT-1 phosphorylation that synergizes with BIM1 down regulation in antagonizing apoptosis. In addition to these subtype-specific abnormalities we have also identified aberrations that have negative impact on the outcome in all DLBCL such as the high expression of the angiogenesis signature, low expression of the MHC signature and *TP53* abnormalities. In follicular lymphoma, we have demonstrated the prominent influence of tumor/host interaction on survival. In contrast, in mantle cell lymphoma, the proliferation signature, which may reflect the convergence of factors dysregulating the cell cycle such as aberrant cyclin-D1 expression and loss of p16/ARF exerted control, is the dominant prognosticator.

These genome scale investigations have helped to unravel multiple oncogenic pathways in the different NHLs. They will continue to provide fresh insight in the future, especially with the addition of high-throughput sequencing that is particularly relevant in B-NHL, as many have aberrant ongoing somatic hypermutation and class switch recombination.

Keywords: lymphoma, gene expression profiling, pathways

063 Deregulation of the Insulin-like Growth Factor Type 1 Receptor (IGF-1R) in Transformed Follicular Lymphomas: Implications for Novel Therapy

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Follicular lymphoma (FL) is the most common form of low-grade non-Hodgkin lymphoma in the western hemisphere. The vast majority of cases are incurable and transformation to diffuse large B-cell lymphoma (DLBCL) is an important cause of death. The molecular and biologic mechanisms underlying FL transformation are largely uncharacterized. In this study, we utilized a global quantitative proteomics approach for the identification of differentially expressed proteins associated with follicular lymphoma transformation.

Five matched pairs of clonally identical cases of follicular lymphoma and their transformed counterparts (DLBCL) arising in the same individual were utilized. Quantitative analysis of differentially expressed proteins were performed by isotope-coded affinity tagging (ICAT^{TM}) followed by liquid chromatography (LC) and tandem mass spectrometry (MS/MS). Equivalent quantities of total cell lysates obtained from the FLs and the DLBCLs were ICAT^{TM} labeled, and subjected to avidin affinity chromatography. Offline fractions were collected, digested with trypsin, and analyzed by automated reverse phase nanospray LC-MS/MS.

Our proteomic studies revealed upregulation of the IGF-1R (3-5 fold) in the transformed lymphomas. Western blot analysis using an antibody to the α-subunit of IGF-1R revealed overexpression in the transformed lymphoma samples as compared to their preceding FL counterparts (discovery set). Similarly, IGF-1R upregulation was demonstrated in an additional independent set of 6/7 DLBCL samples as compared to their preceding FL counterparts. Immunohistochemical studies were performed on formalin-fixed paraffin-embedded tissue sections of 15 matched pairs of FL and their transformed DLBCL counterparts. The neoplastic cells of FL demonstrated negligible levels of IGF-1R whereas the in 5/15 cases, the neoplastic cells of DLBCL demonstrated strong cytoplasmic and membranous expression of IGF-1R. We carried out studies to determine the functional role of IGF-1R in the survival of lymphoma cells in vitro. Blocking antibodies to IGF-1R caused a significant reduction of cell viability in all three transformed FL cell lines (SUDHL-4, OCI-LY1, Karpas 422) as determined by MTT assays. In contrast, antibodies against EGFR, EphA and Frizzled 8 protein did not affect the cell viability of any of the transformed FL cell lines, indicating specificity. Furthermore, knockdown of IGF-1R expression in SUDHL-4 cells by RNA interference resulted in significant reduction in cell viability whereas the control “scramble” siRNA or EGFR siRNA did not have an effect. Cell cycle analysis of the IGF-1R siRNA transfected cells indicated an increase in cells undergoing apoptosis relative to control cells. We utilized a synthetic tyrphostin compound (AG1024) which selectively inhibits the IGF-1R tyrosine kinase activity to determine the effects of pharmacologic inhibition of IGF-1R on the viability of transformed FL cells. Inhibition of IGF-1R resulted in inhibition of cell viability with IC_{50} of 22μM.

This study, for the first time, reveals the role of deregulated expression of IGF-1R in transformed FL and provides a rational basis for the use of IGF-1R blocking agents in the therapy of these neoplasms.

Keywords: proteomics, Lymphoma, mass spectrometry

064 Histiocytic/dendritic Sarcomas Clonally Related to Follicular Lymphoma: Evidence for Lineage Plasticity in Mature Human B-Cells

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Studies in the hematopatholgy section, LP, CCR, can be characterized as Bedside to Bench, in which novel observations made in the clinical setting are translated to the laboratory, and lead to novel insights regarding the pathogenesis of lymphomas. As an example, we have observed rare cases of histiocytic and dendritic cell neoplasms (H/DC) in patients with follicular lymphoma (FL). We studied eight patients with both FL and H/DC neoplasms using immunohistochemistry, fluorescence in situ hybridization (FISH) for t(14;18), and PCR & sequencing of BCL2 and IgH rearrangements.There were 5 men and 3 women (median age, 59y).All cases of FL were positive for t(14;18). Five H/DC tumors were metachronous, following FL by 2 mos to 12 yrs; tumors were synchronous in 3/8 patients. All 7 of the H/DC tumors tested showed the t(14;18) by FISH. In the 8th case the BCL2/JH was confirmed by PCR. PCR for IgH gene rearrangement and the BCL2/JH translocation and sequencing identified identical IgH gene rearrangements or BCL2 gene breakpoints in all patients tested. All H/DC tumors lacked PAX5 and upregulation of CEBPβ and PU.1 was seen in all cases tested. These results provide evidence for a common clonal origin of FL and H/DC neoplasms when occurring in the same patient. Moreover, they provide evidence for transdifferentiation of the FL B-cell clone, possibly without an intermediate step associated with de-differentiation. This report is the first to document lineage plasticity in a mature human B-cell system, and indicates that lineage plasticity is not restricted to immature hematopoietic neoplasms. As previously shown by others in murine systems, the loss of PAX5 appears to be a critical event in alteration of the B-cell program.

Keywords: lymphoma, lineage plasticity, transcription factors

065 Gene Module Regulatory Network Analysis Reveals Stem-Cell Signature in Follicular Lymphoma Transformation

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The transformation of follicular lymphoma (FL) to diffuse large B cell lymphoma (DLBCL) is associated with drastically worse prognosis. Mechanisms underlying transformation are poorly understood, and implicate multiple molecular pathways. To better understand the transformation process for targeted therapy, we constructed a gene module regulatory network from microarray data on clinical samples of FL before and after transformation to DLBCL. Gene modules that significantly discriminate between FL and DLBCL were identified by supervised classification; in addition, genes modules were identified that discriminate FL that are known to transform from FL that do not transform. A network of regulatory gene modules was constructed with a directed edge between pairs of modules if a gene in one module served as a regulator of the other module.

The complete gene regulatory network includes modules that are highly enriched for genes involved in cell cycle progression (False Discovery Rate, FDR=1.2x10-31), proliferation (FDR=2.4x10-25), cellular differentiation (FDR=3.1x10-17), and ribosomal protein activity (FDR=2.8x10-11). Other prominent modules are associated with: immune system function including; T-cell activation and receptor signaling (FDR=9.4x10-10), B-cell development and differentiation (FDR=3.3x10-12), and inflammatory response (FDR=9x10-12); components of the proteasome (FDR=9.3x10-5), hypoxia signaling (FDR=2.5x10-9), and mitochondrial function/oxidative phosphorylation (FDR=4.1x10-6). Known aspects of FL/DLBCL transformation, such as changes attributable to infiltrating T-cell populations were identified and served as internal validation.

The transformation of FL to DLBCL demonstrates changes in cellular differentiation states, proliferative drive, deregulation of mitochondrial function, and increased proteasome activity impacting the cell cycle. Specific modules of genes up-regulated in DLBCL included several related to “stem-cell”-like signatures. These modules were also able to classify subtypes of de novo DLBCL in independent datasets. We find that Pax5 may be a critical factor in transformation of FL; in addition, Bortezamib (a proteasome inhibitor) and Ecteinascidin-743 may be effective agents for FL and transformed DLBCL

Keywords: follicular lymphoma, gene regulatory network, gene expression microarrays

066 The Myeloproliferative Disorders Research Consortium (MPD-RC)

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The Myeloproliferative Disorders Research Consortium (MPD-RC) focuses on the Philadelphia (Ph) chromosome negative MPD including polycythemia vera (PV), and idiopathic myelofibrosis (IM). The goals for the MPD-RC include: 1) Establishment of a multi-institutional international research group entitled the "MPD Research Consortium" which coordinates basic and clinical research dealing with the cellular and genetic foundations of the Ph negative MPD. 2) Establishment of a multi-institutional MPD Clinical Consortium which enables uniform, high volume sample collection, storage and distribution. 3) Performance of rationally designed clinical trials in patients with Ph negative MPD at multiple institutions. 4) Maintenance of an interactive web site for MPD Consortium investigators, a sophisticated international tissue bank and an online database which allows for integration of basic and clinical research. This unique interactive relationship between talented basic researchers and clinical scientists has permitted the MPD Research Consortium to develop novel clinical treatment programs for Ph negative MPD and to identify specific biomarkers that are useful as indicators of therapeutic response and/or risk reduction. The program has six major projects: Project 1: Genetic Basis of Polycythemia Vera; Project 2: Mechanisms and Effects of NF-E2 and PRV-1 Overexpression in PV: Role of Jak2V617F; Project 3: Animal Models of Polycythemia Vera; Project 4: Mouse Models of Myelofibrosis; Project 5: Abnormal Stem Cell Trafficking in Myelofibrosis; Project 6: MPD Clinical Consortium which pursues clinical trials in PV and IM. The six projects are supported by three cores: Core A: Administrative Core; Core B: Biostatistics and Data Management; and Core C: Tissue Bank. These unique interactions between clinical and laboratory investigators which are interwoven within the MPD Research Consortium have lead to an improved understanding of the pathobiology of the Ph negative MPD as well improved strategies which assist in the diagnosis and treatment of these disorders.

Keywords: Philadelphia (Ph) chromosome negative, polycythemia vera (PV), idiopathic myelofibrosis (IM)

067 Studies on Monoclonal Gammopathies

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Multiple myeloma (MM) is an incurable malignancy of plasma cells that evolves from clinically benign precursor conditions, including the premalignant condition monoclonal gammopathy of undetermined significance (MGUS) and an asymptomatic stage called smoldering MM (SMM). The progression from MGUS to SMM and overt, full-blown MM is quite variable. The reason(s) why some patients progress whereas others live out their lives without ever developing MM remains an important clinical question. The overall goal of our program is to elucidate the causes of progression from MGUS → SMM → MM →□ relapsed MM to learn how best to clinically approach each stage of the disease. Our program consists of three projects, a cell and sera collection core, a statistics core, and an administrative core. A major strength of our program is our unparalleled access to primary patient cells and sera from patients with monoclonal gammopathies and our ability to leverage this material toward a better understanding of disease. The cell and sera core began functioning in 1978 and has steadily increased material available for investigation. For example, over the past 12 months, the core collected bone marrow samples from the following patient groups: 45 MGUS, 24 asymptomatic MM, 168 active MM, 70 amyloidosis, 11 macroglobulinemia, and 9 POEMS syndrome. Cells and sera are distributed to a large number of investigators at Mayo, including those leading scientific projects in the program project. In Project 1, Dr. Rajkumar focuses on identifying predictors of progression permitting definition of a subset of MGUS patients with a risk of progression high enough to warrant intervention. In this regard, he has recently demonstrated that the serum free light ratio is a major predictor of outcome in myeloma. He is also performing a family study assessment to determine the prevalence of MGUS in relatives of patients with myeloma and to that end; over 350 patients have already been accrued to this study. The statistics core plays a major role in these epidemiological studies of the monoclonal gammopathies. Lastly, this project also studies the risk of progression from SMM to MM and aims to identify predictors of progression. Dr. Rajkumar recently published a major study of the natural history of SMM and among other factors; levels of serum free light chains were also shown to correlate well with progression. In Project 2, Dr. Jelinek studies mechanisms of myeloma cell growth control with a particular interest in better understanding intraclonal heterogeneity, a hallmark feature of the monoclonal gammopathies. Taking advantage of the rich primary patient resources, her laboratory has had continued success in establishing new models in which to study malignant plasma cell growth and intratumor heterogeneity. She has recently published the establishment and characterization of new cell lines established from the same patient at different stages of disease. Of interest, the patient from which the lines were derived was first diagnosed with primary amyloidosis and then progressed to active myeloma after receiving a stem cell transplant. The two lines together provide an interesting means to study specific events associated with disease progression and also provide the first cell lines known to naturally secrete amyloidogenic light chains. Studies are underway to better understand cellular subsets present in both lines with a primary focus directed toward a better understanding of the biological and genetic features of the cells with extended self-renewal activity. In Project 3, Dr. Fonseca has a major interest in genetic abnormalities in the monoclonal gammopathies including achieving a better understanding of hyperdiploid and non-hyperdiploid MM and MGUS. Of interest, his group has shown that the overall ploidy category in MM is stable over time. He has also used extensive gene expression profiling to determine whether the hyperdiploid variant of MM can be further categorized into other genetic subtypes. This work has led to the identification of a subgroup of patients displaying an NF-kB signature. Information of this nature is vital to identification of new therapeutic strategies that may be effective in this disease. Collectively, our program project continues to be successful in further elucidating the pathophysiology of the monoclonal gammopathies. We believe that these results will ultimately translate into improved and novel therapeutic strategies. Supported by the National Institutes of Health CA062242.

Keywords: myeloma, MGUS, disease progression

068 Use of Genomic Profiling to Identify Prognostic Markers for Ependymomas

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Ependymoma is a central nervous system tumor that affects 170 children per year in North America and carries a discouraging 5-year disease-free survival rate of only 50-60%. The pathogenesis of ependymoma remains poorly defined, and the question of whether anaplastic histology correlates with decreased survival has been controversial in recent retrospective studies. Extent of surgical resection is the only universally accepted prognostic factor. Because of the poor prognosis and the complexity of outcome prediction in ependymoma, clinicians urgently need reliable prognostic markers.

The studies described here were performed as preliminary investigations for the Children's Oncology Group (COG) ependymoma trial ACNS-0121. This trial is the single largest investigation ever undertaken of the genetic bases, treatment alternatives, and clinical outcomes for pediatric intracranial ependymoma. One of the objectives of the COG trial is to identify prognostic and predictive markers for ependymoma by genomic profiling. To meet these objectives, the trial was designed with parallel clinical and biological components. This implementation, in the context of a prospective clinical trial, is ideal for the identification and validation of genetic markers for prognostic and predictive purposes, since clinical outcome and prognosis are therapy-dependent.

In a pilot study using BAC array CGH on 61 cases of intracranial ependymomas, we were able to demonstrate that the location of these tumors (supratentorial vs infratentorial) could be correctly classified based on their genomic abnormalities. In a parallel study using gene expression profiling on 28 cases of intracranial ependymomas, we demonstrated that pediatric intracranial ependymomas can be classified on the molecular level based on both tumor location and histologic grade. We have identified a set of 43 genes that accurately distinguish low-grade ("differentiated") from high-grade ("anaplastic") ependymomas. In addition, we have correlated the gene expression profiles with clinical variables such as patient outcome. Our preliminary studies showed that high expression of ASPM in anaplastic cases exhibits a statistically significant correlation ($p < 0.05$) with decreased event-free survival (EFS). These data supply molecular evidence to support the hypothesis that histologic grade correlates with clinical outcome, and they also provide the foundation for an emerging genomic classification system for ependymoma.

The ACNS0121 study has recently been closed after meeting its accrual goal earlier this year. There were a total of 301 cases with tissues submitted for the genomic study, 17 of which were subsequently declared ineligible after central pathology review. Of the remaining 284 cases, 135 (47.5%) had frozen tissues submitted which will be used for validation of the expression signatures identified in our pilot study. The cases with formalin-fixed, paraffin-embedded (FFPE) tissues will be combined with the remaining frozen tissues in an array CGH study using high density SNP array to complement the expression profiling studies. The expression and CGH results will be integrated in the final analysis and the development of a molecular classification of ependymoma.

Keywords: ependymoma, array CGH profiling, expression profiling

069 Assessment of *in Vivo* Target Inhibition Through the Use of a Plasma Inhibitory Activity (PIA) Assay.

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Kinase inhibitors represent a rapidly expanding class of anti-cancer therapeutics. Their clinical efficacy is dependent upon sustained inhibition of the targeted kinase *in vivo*. Establishing the degree of *in vivo* inhibition, therefore, is a technically challenging but crucial aspect of early phase trials of these agents. We have developed a simple, accurate, and reproducible assay for the quantitative determination of *in vivo* target inhibition by small molecule FLT3 inhibitors. This assay, referred to as the plasma inhibitory activity (PIA) assay, quantifies the amount of FLT3 inhibition induced in a FLT3-expressing cell line incubated with plasma from patients treated with FLT3 inhibitors. The degree of FLT3 inhibition in the PIA assay correlates closely with the actual inhibition determined from direct assay of phosphorylated FLT3 in the circulating leukemia cells from treated patients. This assay allows for quantitative, real-time assessment of target inhibition over multiple time points in patients enrolled on clinical trials of these agents. It complements conventional pharmacokinetics because it measures the biologic activity of a compound, rather than just the concentrations of parent drug and metabolites. This allows for greater confidence in the selection of an optimal dose for phase II studies. Finally, the assay is applicable to any small molecule targeted agent for which a target can be assessed in a cell-based model system. We are currently using this assay to establish the efficacy of *in vivo* target inhibition in 5 ongoing clinical trials of FLT3 inhibitors. The PIA results from these trials will be presented both as a demonstration of the utility of this assay technique as well as to provide an update on the relative *in vivo* potency of different FLT3 inhibitors currently being investigated.

Keywords: AML, Kinase, FLT3

070 Whole Genome Sequencing of an Acute Myeloid Leukemia Genome with Normal Cytogenetics

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For most patients with a sporadic presentation of acute myeloid leukemia (AML), neither the initiating nor the progression mutations responsible for disease are known. Recent attempts to identify key mutations with directed sequencing approaches, or with array-based genomic studies, have had limited success, suggesting that unbiased whole genome sequencing approaches may be required to identify most of the mutations responsible for AML pathogenesis. Until recently, whole genome sequencing has been impractical due to the high cost of conventional capillary-based sequencing, and the large numbers of enriched primary tumors cells required to yield the necessary genomic DNA for library preparation. "Next Generation" sequencing approaches have changed this landscape dramatically. Using the Solexa/Illumina platform, we have now sequenced the genomic DNA of highly enriched tumor cells and normal skin cells obtained from a carefully selected patient with a typical presentation of FAB M1 AML. We obtained 98.2 billion bases of sequences from the cytogenetically normal tumor cell genome (32.7 fold haploid coverage), and 41.8 billion bases of sequence from the normal skin genome (13.9 fold coverage). Using these data, we detected diploid sequence coverage of 91.2% of 46,494 heterozygous SNPs defined in the tumor genome (by array-based genotyping) and 82.6% diploid coverage of the skin genome. Of 2,647,695 well-supported single nucleotide variants detected in the tumor genome, 2,588,486 (97.7%) were also detected in the patient's skin genome, defining them as inherited. Of the 59,209 tumor-specific variants, 29,761 were novel (i.e., not in dbSNP or the Watson/Venter genomes). We focused on the 171 predicted non-synonymous changes in the coding region of genes, since their potential biological consequences are the easiest to understand and they provide the shortest path to clinical translation. Using traditional capillary sequencing chemistry, we confirmed eight novel heterozygous somatic mutations in this patient's tumor sample. We also detected somatic mutations in the FLT3 (ITD) and NPM1 genes (a classic NPMc mutation). None of the eight novel mutations identified thus far have previously been detected in AML cases (and none were found in any of 187 additional AML cases studied here). Based on deep readcount data (454/FLX), we determined that all of these mutations (except FLT3) were present in virtually all tumor cells at presentation, and again at relapse 11 months later, suggesting that the patient had a single dominant clone containing all of the mutations. A number of additional potential somatic mutations in regions lying near genes (but not altering coding sequences) are currently being validated and tested for recurrence in other AML samples. Whole genome sequencing of a second M1 AML genome is now underway. These results demonstrate the power of unbiased whole genome sequencing approaches to discover cancer-associated mutations in novel candidate genes.

Keywords: acute myeloid leukemia, Next-generation DNA sequencing, molecular genetics

071 Frequency of Mitochondrial DNA and Hemochromatosis Gene Mutations in Survivors of Pediatric Acute Lymphoblastic Leukemia at Risk for Anthracycline-Cardiotoxicity

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The cardiovascular health of long-term survivors of childhood cancer is particularly compromised following anthracycline chemotherapy. Congestive heart failure, coronary artery disease and cerebrovascular accidents are each significantly more common among childhood cancer survivors than the general populations.

Animal models and preliminary evidence implicate anthracycline-iron complexes and mitochondrial dysfunction related to free-radical activity in anthracycline-related cardiomyocyte damage. In the current study, we sought to extend understanding of anthracycline-related cardiotoxicity through a translational study of mitochondrial DNA (mtDNA) and hemochromatosis gene (HFE) mutations and cardiovascular risk among a well-described group of long-term survivors of high-risk childhood acute lymphoblastic leukemia (ALL). We also examined whether dexrazoxane (a cardioprotective free-radical scavenger) reduced the frequency of mtDNA mutations. Participants were enrolled 4 or more years post high-dose (300 mg/m^2) doxorubicin treatment. We quantitatively assessed the frequency of mtDNA mutations in peripheral blood mononuclear cells via denaturing gradient gel electrophoresis (DGGE). To detect the most common mutations in hereditary hemochromatosis (cysteine to tyrosine at amino acid position 282 (C282Y), the histidine to aspartic acid at amino acid position 63 (H63D)) or the serine to cysteine at amino acid position 65 (S65C), we directly sequenced genomic DNA using a commercial clinically validated kit available from Biotage Inc (PyroMark HFE Cat#40-0053) run on a Pyrosequencer instrument (PSQ HS 96, Biotage Inc). Serial echocardiography and biomarker serology (proBNP, hsCRP, serum lipids and homocysteine) assessed cardiac function.

Preliminary results suggest that the rate of mtDNA mutations and rare polymorphisms is significantly more common among survivors of childhood leukemia than is expected based on results from healthy children ($\geq$ 1 sequence variant: 22/62 LTS vs.7/56 healthy newborns, $p = 0.0025$). Dexrazoxane did not appear to reduce sequence variant frequency. The frequency of HFE mutations is similar to the general population; 10% of patients screened to date are heterozygous for a HFE C282Y mutation (~6-9.5% allelic frequency), 18% are heterozygous for a H63D mutations (~13.5% allelic frequency), and 3% are heterozygous for a HFE S65C mutation. Two homozygotes and 1 compound heterozygote have also been identified. Cardiac assessment is ongoing.

Keywords: doxorubicin, cardiotoxicity, acute lymphoblastic leukemia

072 Silvestrol: A Novel B-Cell Selective Agent for Potential Use in Leukemias and Lymphomas

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Therapeutic options for advanced B-cell malignancies including acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) are limited. Furthermore, currently available treatments can deplete T lymphocytes, resulting in life-threatening opportunistic infections. Agents with novel mechanisms of action and B-lymphocyte selectivity are needed in both diseases. The structurally unique natural product silvestrol was identified through an NCI National Cooperative Drug Discovery Group (Kinghorn, PI). In the NCI 60-cell line screen, silvestrol produces a unique pattern of cytotoxicity suggesting unusual mechanism and efficacy in leukemia. Using primary human leukemia cells, established leukemic cell lines, and animal models, we assessed silvestrol for its potential as a B-cell selective anti-cancer therapy. In CLL patient tumor cells, the silvestrol LC_{50} (concentration lethal to 50%) is 6.5 nM at 72 hours. At this concentration, there is no difference in silvestrol sensitivity of tumor cells from CLL patients with or without the 17p13.1 chromosomal deletion (*p53* site). In isolated cells and in whole blood, both from healthy volunteers and CLL patients, silvestrol is significantly more cytotoxic toward B cells than T cells. Silvestrol causes early reduction in the anti-apoptotic protein Mcl-1 due to translational inhibition with subsequent caspase-independent loss in mitochondrial membrane potential. *In vivo*, silvestrol causes significant B cell reduction relative to T cells in Tcl-1 transgenic mice, and extends survival of 697-SCID mice without discernable toxicity. These data indicate silvestrol has unusual potency and B-cell selectivity both *in vitro* and *in vivo*, and support its development for B-cell malignancies. Due in part to these findings, silvestrol is now undergoing preclinical evaluation by the by the NCI Developmental Therapeutics Program's Drug Development Group at the Stage IIA level.

Keywords: leukemia, drug development, natural product

073 Biomarkers for Risk and Target Identifications in Pediatric AML

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National Childhood Cancer Foundation

The Children's Oncology Group (COG) is an international clinical trials cooperative group supported by the National Cancer Institute with the unique ability to enroll nearly all pediatric patients in North America as well as those in Australia, New Zealand and select European countries in its clinical trials, resulting in standard of care through state of the art treatments for children with cancer. As part of efforts to optimize outcome in children with acute myeloid leukemia (AML,) COG Myeloid Disease Committee has adopted a risk-based therapy in AML in order to decrease relapse in high-risk patients and minimize toxicity in those expected to have a more favorable outcome. Identification of biomarkers for risk and therapeutic target identification has been incorporated into COG AML phase III trials. As part of these efforts, FLT3/ITD with high allelic ratio (high ITD-AR) has been identified as a marker of high-risk disease in AML and validated in an independent European pediatric trial (DCOG). As a result, high ITD-AR has been incorporated as a clinical risk factor in the current COG phase III AML trial, where those with high ITD-AR will be allocated to receive allogeneic stem cell transplant in first CR, with plans to incorporate FLT3 inhibitors into their therapy following current feasibility testing. Comprehensive molecular profiling for the purpose of risk and target identification is underway, including CEBPA, NPM, WT1, MLL/PTD, as well as RAS and RAF gene alterations. We have demonstrated that those with CEBPA mutations have lower relapse risk and improved outcome. We are validating these findings in the current trial (AAML0531) and will incorporate this risk factor in the next phase III trial (COG AAML0931).

Further efforts are being focused on post induction evaluation of response to therapy as a means of risk identification. Recent data generated as part of COG AAML03P1 validated the role of next generation multidimensional flow cytometry (MDF) based MRD in identification of those in morphologic remission who remain at high risk of relapse. As an integrated aim in the current trial, we will optimize the utility of MDF (threshold, time point, etc) to identify patients in morphologic remission with minimal residual disease (MRD) who are at high risk of relapse. Additional efforts are underway for evaluation of molecular MRD in those with specific cytogenetic markers, including t(8;21), inv(16) and t(9;11). MDF based MRD will be merged with molecular MRD, cytogenetic profiles and mutational profile and will be collectively assessed in order to define the most accurate risk profile for the largest number of patients. The data generated will be used for risk identification as part of risk-based therapy in the next phase III trial.

Keywords: molecular genetics, risk adjusted therapy/pronostic classification, molecularly targeted therapy

074 Ad-sTRAIL, an Adenoviral Construct Expressing Soluble TRAIL, Improves Survival in a Bioluminescence Imageable Intracranial Xenograft Model and an *ex-Vivo* Model Of Malignant Glioma

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Malignant cells exhibit resistance to apoptosis and selective activation of pro-survival pathways that enable them to proliferate and survive in adverse conditions. However, many malignancies express receptors to Apo2L/TRAIL (TNF-α related apoptosis inducing ligand), a death inducing ligand that selectively induces apoptosis in tumor cells. Being a soluble ligand, TRAIL may potentially be a suitable agent for therapy of locoregional tumors such as malignant gliomas. We have previously demonstrated that TRAIL can robustly induce apoptosis in glioma cells and can interact with the Akt-mediated survival pathway.

In this study, we generated an adenoviral construct that expresses the soluble extracellular portion of TRAIL (Ad-sTRAIL) and tested its *in vitro* activity and in vivo activity. We also tested the *in vivo* effects of this agent against an intracranial glioma xenograft model in nude mice using U251HF and SNB19 glioma cells stably transfected with luciferase allowing bioluminescent imaging of tumor growth with intratumoral Ad-sTRAIL injections given twice weekly for 4 weeks with injection of Ad-EGFP and PBS as controls. Tumors formed by SNB19 and U251HF cells displayed characteristics of human high grade gliomas including high cellularity, pleomorphism, vascular proliferation and necrosis. Ad-sTRAIL and Ad-EGFP treated tumors showed adenoviral hexon protein expression. Tumors treated with Ad-sTRAIL, but not Ad-EGFP or PBS, showed caspase 3 activation and TUNEL positivity by immunohistochemical staining indicating selective induction of apoptosis by the agent. Bioluminescent imaging at days 7, 14, 21 and 28 demonstrated unrestricted growth of tumors in the PBS and Ad-EGFP treated animals but strong inhibition of tumor growth in the Ad-sTRAIL treated tumors. Ad-sTRAIL treated animals also showed prolonged overall survival compared with control animals suggesting that this agent has potential for clinical activity against malignant gliomas.

To further examine these effects in human tissue, we developed a human glioblastoma ex-vivo organotypic slice culture model; Upon treatment with Ad-sTRAIL, human glioma slices showed induction of apoptosis and caspase activation. In addition, we studied the effect of inhibition of XIAP, a potent inhibtor of death receptor mediated apoptosis, on TRAIL sensitivity in the U251HF intracranial glioma model. Intratumoral treatment with Ad-XAF1 (expressing XAF1 which binds and inhibits XIAP inhibitor) prior to treatment with Ads-TRAIL, resulted not only in durable tumor responses but also significantly improved survival compared with Ads-TRAIL alone. Our results suggest that additional studies of Ad-sTRAIL, in combination with strategies overcoming potential resistance mechanisms of death receptor induced apoptosis, against malignant gliomas are warranted to fully explore its potential as a therapeutic agent.

Keywords: apoptosis, TRAIL, glioma

075 Development and Validation of a Highly Accurate Classifier for CTCL Using Microarrays and Q-RT-PCR

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Primary Cutaneous T-cell Lymphomas (CTCLs) are a heterogeneous group of non-Hodgkin's lymphomas, characterized by the clonal proliferation of malignant, skin-invasive $CD4^+$ helper T-cells with Mycosis Fungoides (MF) and Sézary Syndrome (SS) being the most commonly occurring CTCL subtypes. MF can slowly progress to an advanced stage that can include the development of multiple tumors, erythroderma, blood and/or organ involvement. SS the leukemic, aggressive form of the disease is characterized by acute erythroderma, circulating malignant T-cells with cerebriform nuclei and a median survival time of 2 to 4 years. These patients are characteristically resistant to conventional chemotherapy. Our initial studies focused on SS patients.

When gene expression data for patients with high blood tumor burden (Sezary cells>60% of the lymphocytes) and Th2 skewed PBMC controls were compared, 385 genes were found to be differentially expressed at $p<.01$ with a False Discovery rate (FDR) of 8% and an accuracy >95%. Highly over expressed genes included Th2 T-cell–specific transcription factors Gata-3 and Jun B, as well as integrin β1, proteoglycan 2, the RhoB oncogene, and dual specificity phosphatase 1, supporting the Th2 character of SS cell. Highly underexpressed genes included *CD26, Stat-4*, and the *IL-1* receptors. We had previously shown that Stat 4 was underrepresented in these patients [1] and the microarray studies confirmed the effect on the transcriptional level in an expanded patient group. We also identified a new CTCL marker Plastin-T (PLS-3), not normally expressed in lymphoid cells, which is single gene marker for CTCL diagnosis that is informative for approximately 75% of patients tested. Using penalized discriminant analysis (PDA), we identified a panel of eight genes developed on the high tumor burden patients that also distinguished 27 SS in patients some with as few as 5% circulating malignant T-cells, the threshold for pathological diagnosis, from 14 controls with 100% accuracy. This suggests that, even in early disease, Sezary cells produce chemokines and cytokines that induce an expression profile in the peripheral blood, including normal lymphocytes, that is specific to SS. Finally, we demonstrated that a panel of 10 genes identified a class of patients who succumbed within six months of sampling that was independent of their tumor burden [2].

We further validated the microarray findings on new samples using a quantitative real-time polymerase chain reaction (QRT-PCR) assay. Expression values for just 5 of those genes, STAT4, GATA-3, PLS3, CD1d and TRAIL determined using QRT-PCR data from PBMC accurately classified 88% of 17 patients with high blood tumor burden and 100% of 12 healthy controls in the training set using Fisher Linear Discriminant Analysis (FLDA). The same 5 genes were then assayed on 56 new samples from 49 SS patients with blood tumor burdens of 5-99% and 69 samples from 65 new healthy controls. The samples were derived from 4 different locations. The average accuracy in the independent validation set over 1000 re-samplings was 90% using FLDA and 88% using Support Vector Machine (SVM).Including PLS3 positivity brought the accuracy to 96%. We also tested the 5 gene PCR classifier on 14 samples from MF patients with no detectable peripheral blood involvement and 3 patients with Atopic Dermatitis (a Th2 disease) with severe erythroderma. They were classified as non-SS patients with 100% accuracy [3]. This 5 gene signature is being monitored in CTCL patients in clinical trials at NCI and the University of Pennsylvania with the HDAC inhibitor Romodepsin to develop a quantitative assessment of response to therapy.

References: 1. Showe, L.C., et al., J Immunol, 1999. 163(7): p. 4073-9. 2. Kari, L., et al., J Exp Med, 2003. 197(11): p. 1477-88. 3. Nebozhyn, M., et al., Blood, 2006. 107(8): p. 3189-96.

Keywords: microarrays, PCR, classification

076 Biology and Therapy of High-Risk Neuroblastoma

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Neuroblastoma is the most common extra cranial solid tumor of childhood, and 45% of patients have aggressive tumors, most of which are metastatic (stage 4) when diagnosed. Previous studies have demonstrated that both intensive cytotoxic therapy with autologous hematopoietic progenitor cell transplant and post-transplant 13-cis-retinoic acid improve outcome. For example, in the recently completed Children's Oncology Group (COG) A3973 study, 49% of patients were event-free survivors and 73% were surviving two years after diagnosis. However, the limit of host tolerance for intensive, non-targeted cytotoxic therapy likely has been reached. The overall **hypothesis** of our Program Project Grant (5 P01 CA81403-09) is that improved survival requires an understanding of the biology of tumor cells, host cells, and of their interactions and the development of therapies that target pathways that are critically important for neuroblastoma growth. The **aims** of the Projects in this PPG are to integrate biologic and preclinical therapeutic research with early phase clinical trials. Project 1: The tumor microenvironment is investigated with emphasis on tumor cell, bone marrow mesenchymal cell, and osteoclast interactions in bone metastasis. Neuroblastoma cells were shown to produce galectin-3 binding protein, which induces mesenchymal cells to secrete IL-6, which, in turn, activates osteoclasts that enhance formation of bone metastasis. A bisphosphonate, zoledronic acid, markedly inhibits osteoclast activity and bone invasion by tumor cells, and this agent was tested for the first time in children by our New Approaches to Neuroblastoma Therapy (NANT) consortium. Project 2: Natural killer (NK) cells alone and with an anti-disialoganglioside antibody (anti-GD2 mAb) were shown to kill drug-sensitive and -resistant neuroblastoma cell lines in vitro. Using neuroblastoma metastatic models in NOD/SCID mice, NK + mAb therapy, if initiated before metastases are detectible by bioluminescent imaging, can cure 65% of mice (minimal residual disease model). However, when disease is detectible, immunotherapy, although increasing survival time, is most effective if combined with other agents (bolus and metronomic cyclophosphamide, bevacizumab, zoledronic acid). Some stage 4 MYCN non-amplified tumors from patients were found to have high levels of gene expression representing immune/inflammatory cells (B cells, macrophages) and cytokines (IL-6, IL-10, TGFβ1), which correlated with <20% progression-free survival. The effects of such a tumor promoting microenvironment on NK + mAb cytotoxicity are being determined. Project 3: The cytotoxic retinoid, fenretinide was shown to increase ceramide induction and tumor cell death. Agents that synergize with fenretinide to further enhance ceramide mediated cytotoxicity have been identified based upon ceramide pathway analyses. A new oral liquid formulation of fenretinide that was developed in collaboration with the NCI RAID program was tested by the NANT consortium and is superior to an earlier capsule formulation with respect to obtaining potentially effective drug levels. Project 4: New strategies targeting tumor and/or normal microenvironment cells are tested in phase I and II trials by the NANT consortium (www.nant.org), which includes 14 pediatric oncology institutions across the US and in Canada and which accrues 55 patients annually. Fourteen clinical trials have been opened through 2007 with four being completed and eight currently active. Approaches that are promising are moved forward in the clinical trial pipeline. For example, targeted radiation from ^{131}I-metaiodobenzylguanidine (MIBG) combined with high dose chemotherapy has been tested in phase I and II trials by the NANT and is now being developed for newly diagnosed patients by the COG. In **summary**, a continuum of integrated pre-clinical and early phase clinical research is providing new insights and therapeutic strategies for achieving our ultimate goal of improving survival for children with high-risk neuroblastoma.

Keywords: neuroblastoma, drugs, biologics

077 DNA Methylation as Biomarkers in Non-Hodgkin's Lymphoma

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Beyond genetic alterations in various types of cancer, epigenetic alterations are now known to play a very important role in cellular dysregulation. One of the main epigenetic modifications involves methylation of cytosine molecules within regulatory and repeat sequences of tumors that do not exist in normal cells, with few exceptions. We initially undertook discovery-based studies of DNA methylation patterns using microarray technologies for the purpose of discovering large-scale abnormalities in methylation patterns in certain subclasses of non-Hodgkin's lymphomas compared to normal lymphoid cells. Over time, we have moved from use of very early-stage microarrays that used crude DNA fragments enriched for gene promoter regions, but have now moved to used of much more robust oligonucleotide microarrays and DNA sequencing technologies. Our key findings are that: 1.) All these technologies are useful in different ways; 2.) Non-random patterns of DNA methylation allow us to segregate different subclasses of NHL; 3.) Methylated DNA circulating in blood shows the same patterns of methylation as in the primary tumor; 4.) Whether using DNA from the primary tumor or from blood plasma, patterns of DNA methylation show promise in differential diagnosis, tumor subclassification, and potentially for monitoring successful treatments. This project then has moved from early discovery to identifying candidate biomarkers that can be used in translational medicine to improve patient care.

Keywords: methylation, epigenetics, lymphoma

078 Gene Expression Classifiers for Minimal Residual Disease (MRD) and Relapse Free Survival (RFS) Improve Outcome Prediction and Risk Classification in Children with High Risk Acute Lymphoblastic Leukemia (ALL): A Project of the Children's Oncology Group and the NCI Strategic Partnerships to Evaluate Cancer Gene Signatures (SPECS) and NCI TARGET Programs

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Background: Remarkable advances in treatment outcome have been achieved in the majority of children with ALL through the optimization and progressive intensification of chemotherapeutic regimens employing cytotoxic agents that have been in widespread clinical use since the early 1970s. In parallel, laboratory investigations have provided remarkable insights into the underlying genetic heterogeneity and molecular pathogenesis of this disease, with the characterization of many frequently recurring genetic abnormalities that have prognostic import and which are associated with distinct clinical phenotypes. These studies have identified a number of distinct molecular subtypes of pediatric ALL that are associated with treatment outcomes that are either excellent (such as TEL-AML1 or trisomies of chromosomes 4, 10, and 17 in children with "low risk" disease) or very poor (such as BCR-ABL or hypodiploidy in children with "very high risk" disease). Yet, these genetic abnormalities account for only 35-40% of all cases of pediatric ALL. The underlying genetic abnormalities in the majority of children with ALL, such as those with "high risk" disease who have remained resistant to current therapies, remain to be discovered. The goal of our studies has been to use gene expression profiling in high risk ALL to: 1) develop molecular classifiers for outcome prediction that can be used to improve risk classification and therapeutic targeting; 2) incorporate these classifiers into COG clinical trials; and 3) discover novel cluster groups and underlying genetic abnormalities to in order to identify potential new therapeutic targets. **Methods:** Expression profiles were obtained in pre-treatment leukemic samples from 207 uniformly treated children with high risk ALL treated in COG/POG Trial 9906. Relapse free survival (RFS) was 61% at 4 years and flow cytometric measures of minimal residual disease (MRD) at the end of induction (day 29) were predictive of outcome ($P<0.001$). Molecular classifiers predictive of RFS and MRD were developed using extensive cross-validation procedures. **Results:** A 38 gene molecular risk classifier predictive of RFS (MRC-RFS) distinguished two groups of high risk ALL patients with different relapse risks: low (4 yr RFS: 81%, n=109) vs. high (4 yr RFS: 50%, n=98) ($P< 0.0001$). In multivariate analysis, the best predictor combined MRC-RFS and day 29 flow MRD data, classifying children into low (87% RFS), intermediate (62% RFS), or high risk (29% RFS) groups ($P<0.0001$). A 21 gene molecular classifier predictive of MRD could effectively substitute for day 29 flow MRD, yielding a combined classifier that similarly distinguished three risk groups at pre-treatment (low: 82% RFS; intermediate: 63% RFS; and high risk: 45% RFS) ($P< 0.0001$). This combined molecular classifier was further validated on an independent cohort of 84 children with high risk ALL registered to COG/CCG Trial 1961 ($P = 0.006$). Using 4 unsupervised clustering methods, we defined 8 distinct cluster groups. While 2 cluster groups contained those cases with t(1;19)/E2A-PBX translocations or MLL rearrangements, the other 6 cluster groups were completely novel. Two of the novel cluster groups had strikingly different outcomes (95% 4 year RFS vs 20% 4 year EFS) and were associated with distinct novel underlying genetic abnormalities and genes that may represent novel therapeutic targets. Children of Hispanic/Latino ethnicity were disproportionately present in the poorest outcome cluster. **Conclusions:** Molecular classifiers can be used to distinguish distinct prognostic groups within high risk ALL, significantly improving risk classification schemes and the ability to prospective identify children who will respond to or fail current therapies. These classifiers are now being integrated into the design of COG trials. The discovery of novel underlying cluster groups and genetic abnormalities is being used to develop novel therapeutic targets.

Keywords: leukemia, gene expression profiling, molecular classifiers

079 Using Custom Protein Microarrays to Identify Autoantibody Biomarkers for the Early Detection of Cancer

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Background: Cancer patients make antibodies to tumor-derived proteins that are potential biomarkers for early detection. To detect autoantibodies to tumor antigens in patient sera, we have adapted novel high-density custom protein microarrays (NAPPA) expressing 6,500 candidate tumor antigens for biomarker detection. These arrays are probed with sera from patients with early stage breast cancer and healthy women. Using this approach, we identified antibodies in the sera of breast, ovarian, and prostate cancer patients.

Methods: 6,500 full-length human antigens were expressed using mammalian reticulocyte lysate and captured onto NAPPA protein microarrays. Protein expression (>90%) was confirmed with anti-GST antibodies. Patient sera were added, and bound IgG detected with secondary antibodies. Serum samples were obtained from 53 patients with stages I-III breast cancer and 53 age-matched controls, 15 patients with stages I-III ovarian cancer and 15 controls, and 25 prostate cancer patients and 17 controls.

Results: Using high-density protein microarrays, sera from cancer patients (n=93) and healthy donor sera (n=85) were screened for autoantibodies to up to 6,500 protein antigens. Antigens were selected for further analysis if the 95th percentile of signal of cases and controls were significantly different ($p<0.05$) and if the number of cases with signals above the 95th percentile of controls was larger than the number expected due to random chance ($p<0.05$). Known tumor antigens, such as p53, were detected, as well as novel antigens such as DCC1, Rab7L and DCP1A. In total, up to 768 unique antigens were selected for each disease for further analysis with independent sets of sera.

Conclusions: Custom in-situ protein microarrays can be used to detect serum tumor antigen-specific antibodies and enables the rapid, simultaneous detection of immunogenic tumor antigens from patient sera. These autoantibodies are being evaluated as potential biomarkers for the early diagnosis of cancer.

Keywords: biomarkers, breast cancer, ovarian cancer

080 Multiplex Serum Analysis for Screening and Diagnosis of Pancreatic Cancer

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Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. Currently, no effective early detection and screening modalities are available and tumors are typically diagnosed at a late stage, frequently after metastasis. Identification of molecular biomarkers for early detection and correct diagnosis may significantly improve the survival of patients with pancreatic cancer. None of known biomarkers of pancreatic cancer including CA 19-9 are sensitive and specific enough to be used singly for screening or diagnosis of the disease. We hypothesized that combination of several serum biomarkers representing various aspects of pancreatic cancerogenesis would offer higher classification power compared to single biomarkers. We have utilized a bead-based immunoassay approach that allows simultaneous measurement of multiple markers. In this study, a panel of 90 serological markers including cytokines, chemokines, growth and angiogenic factors, hormones, proteins associated with metastasis and apoptosis, and others in combination with CA 19-9 was analyzed in sera of 360 pancreatic cancer patients, 127 patients with chronic pancreatitis, and 686 matched control healthy subjects obtained from three different institutions, Evanston Northwestern Healthcare, Memorial Sloan-Kettering Cancer Center, and University of Pittsburgh Cancer Institute. Subset obtained from Memorial Sloan-Kettering Cancer Center (133 cancer and 72 benign cases) was used as a training set, and the rest of the samples were used as a blinded validation set. Bioinformatics analysis identified a panel consisting of CA 19-9, CEA, and ICAM-1 that was able to distinguish pancreatic cancer from benign disease with a sensitivity of 77% and specificity of 91%, which was superior to performance of CA 19-9 alone. This panel when validated in a blinded validation set offered sensitivity of 75% at a specificity of 90% for discrimination of pancreatic cancer from benign cases with the concomitant specificity of 91% for healthy controls. In conclusion, we have identified and validated a serum multimarker panel, which was able to discriminate pancreatic cancer from benign pancreatic condition and from healthy controls with high sensitivity and specificity.

Keywords: pancreatic cancer, serum biomarkers, disgnosis

081 The TCGA Cancer Genome Characterization Center (CGCC) at MSKCC: High Resolution Genomic Copy Number Analysis of the First 215 Glioblastoma Samples

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Objectives: Within The Cancer Genome Atlas (TCGA) consortium, the MSK-CGCC performs comprehensive profiling of genomic copy number alterations (CNAs) using Agilent 244K CGH arrays, followed by bioinformatic analyses to identify candidate genes for resequencing by the TCGA Genome Sequencing Centers. Furthermore, we have designed a complementary custom Agilent array based on a cancer gene-centered strategy optimally suited for the high resolution detection of intragenic CNAs that may represent intragenic deletions, either activating (e.g. EGFRvIII) or inactivating (e.g. PTEN), or intergenic rearrangements leading to the formation of fusion oncogenes.

Methods: Two distinct CNA analysis algorithms were developed by MSK-CGCC members, GTS (Genome Topography Scan) and RAE. The custom high density CGH array was designed for densest coverage (~400bp) of tyrosine kinases (n=86), transcription factors (n=823), and genes with suspected intragenic CNAs (n=103) in data from standard Agilent 244K CGH arrays. Another 5170 genes, covered at ~1.5kb intervals, were selected bioinformatically based on literature and motif data. A statistical approach to identifying significant intragenic CNAs was developed based on background rate, event recurrence, and gene size.

Results: Unsupervised analysis showed 3 patterns of broad CNAs in these 215 GBMs, with two of the clusters being driven by coordinate gains of chromosomes 19 and 20 and losses of 13 and 14, respectively, and 9p (CDKN2A) and chromosome 10 (PTEN) losses being present in all 3 clusters. Narrow CNAs included gains in EGFR, MET, MDM2, CDK4, PDGFRA, KIT, KDR, and MDM4, and losses in PTEN, CDKN2A/B/C, NF1, RB1, PTPRD, and FBXW7, among others. Among intragenic CNAs, EGFR was the top gene, showing the expected EGFRvIII intragenic deletions involving exons encoding the extracellular domain as well as other deletion mutations. Integration of intragenic CNAs with matching Affymetrix Exon array data (generated by another TCGA CGCC) identified 82 genes in which the CNA breakpoint was paralleled by an intragenic change in exon expression levels. These are lead candidates for RACE or other sequencing strategies to identify putative fusion partners.

Conclusion: The GTS and RAE analysis algorithms generate very similar data for the major recurrent CNAs in GBM, and use different strategies to tackle the challenge of assessing the significance of less frequent narrow events. This analysis limited to a single genomic platform within the TCGA and covering only the first 215 samples of up to 500 anticipated for this tumor type is already yielding an unprecedented portrait of somatic chromosomal aberrations in GBM.

Keywords: microarray, genomic profiling, translocation

082 Comprehensive Transposon Mapping in the Human Genome

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Transposons are mobile genetic elements which have shaped eukaryotic genomes over evolutionary time. Subsets remain animated in our modern human genomes, contributing to genetic diversity, heritable disease, and oncogenesis to degrees not well understood. Some of the most dynamic transposons are LINEs, which multiply in the genome by "copy-and-paste" through an RNA intermediate. They have been difficult to study because: (*i*.) LINE prevalence in the genome precludes accurate copy number quantification, (*ii*.) LINE transcription cannot be accurately assessed by RT-PCR, Northern blots, or expression microarrays, (*iii*.) there are no robust, widely-available antibodies against LINE-encoded proteins, (*iv*.) many genome projects rely on sequencing strategies that fail to detect full-length, 6 kb LINE insertions and non-exonic insertions, and (*v*.) until now, no method for finding new insertions in the vastness of the genome was feasible. The Boeke laboratory previously reported a method to map Ty transposons in yeast by coupling a transposon insertion profiling (TIP) PCR with microarray amplicon analysis (TIP-chip). We now demonstrate that this approach can be applied to the human genome to identify positions of active T(a)LINE retrotransposons. The PCR strategy amplifies genomic DNA flanking T(a)LINE integration sites, and shows excellent discrimination between T(a)LINEs and older inactive LINEs. In addition to allowing genotyping for known T(a)LINE insertions, limited application of the technique has demonstrated numerous novel polymorphic insertions, as well as candidate disease-causing insertions in patients with heritable diseases whose specific sequence defects have been elusive. The technique is being scaled for comprehensive genome coverage for the evaluation of transposon instability in the development of solid tumors and leukemias. We expect this will add a new dimension to our understanding of structural variation in the human genome and provide new insights into human disease.

Keywords: transposon, T(a)LINE, microarray

083 Measuring Cancer Biomarker Candidates by Targeted MS and Ab Enrichment

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Better biomarkers are urgently needed to improve diagnosis, guide molecularly targeted therapy, and monitor activity and therapeutic response across a wide spectrum of disease. Proteomics methods based on mass spectrometry hold special promise for the discovery of novel biomarkers that might form the foundation for new clinical blood tests, but to date their contribution to the diagnostic armamentarium has been disappointing. That disappointment is not due to a lack of biomarker candidates, but rather the inability to rapidly and accurately follow-up on the large numbers of candidates that are generated. Unbiased proteomic discovery in tissue or proximal fluids using a processing pipeline consisting of abundant protein depletion, multidimensional fractionation of peptides and LC-MS/MS and modern, high performance hybrid MS systems (e.g., Orbitraps) yields 1000's of candidate proteins, a high percentage of which show differential expression in cases vs. controls. The lack of robust quantitative methods with sufficient sensitivity, reproducibility and throughput has significantly hampered our ability to credential these candidates coming from unbiased proteomic discovery efforts.

Our group, funded by the NCI CPTAC Program, is focusing our efforts in addressing this serious barrier by developing robust, multiplexed targeted screening methods as well as quantitative assay methods for candidate biomarker proteins in plasma. Candidate proteins prioritized by integrative genomics or AIMS (an MS-based technology recently developed at the Broad, see ref. 1) moved into quantitative assay development by "multiple reaction monitoring" MS (or MRM) using stable-isotope dilution, a well-established quantitative MS technique used in the routine measurement of drugs and metabolites. This approach is quite distinct from conventional proteomic methods in that the mass spectrometer is forced to focus on and measure only specific signals derived from the candidate proteins. This overcomes a major limitation of mass spectrometers as well as humans, in that we both have a limited attention span and can only tolerate so much information at once. The MRM approach is highly specific, quantitative and can be multiplexed to a very high level enabling 100's of candidate protein biomarkers be tracked in a single experiment. We have demonstrated that it is now possible to configure multiplexed assays for proteins at the low ng/mL level in plasma by combining simple depletion and fractionation approaches with detection by multiple reaction monitoring on triple quadrupole MS systems (2). Further improvements may come from the use of peptide immunoaffinity enrichment which holds particular promise for simplifying sample preparation and increasing both throughput and sensitivity of MRM-based assays. Using SISCAPA, assays can be readily configured that enable quantitation of proteins present at low ng/mL levels directly from plasma (3). After extensive optimization and characterization, our assays will be deployed on clinical plasma samples from breast cancer patients to measure protein biomarker levels in a few hundred selected cancer/control plasma samples. The results will characterize the quantitative reproducibility of the assay methodology within- and between-laboratories, and over time. We expect to deliver to the community a series of standardized reagents and developed assays that can be implemented in other laboratories using similar standardized instrumentation.

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Keywords: multiplexed quantitative assay, targeted mass spectrometry, protein biomarker verification and validation

084 Translation of Proteomic Biomarker Discovery Into Clinical Practice for the Early Detection of Cancer

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Currently for all cancer types, there are only a dozen serum cancer proteomic biomarkers cleared/approved by the US FDA for clinical use. While many more candidate biomarkers have been reported in the scientific literature, very few biomarkers have been validated and developed into diagnostics. The Early Detection Research Network (EDRN) Biomarker Reference Laboratory at The Johns Hopkins University was established to facilitate the translation of cancer biomarkers from discovery to clinical practice. We have assembled a multi-disciplinary team of scientists and developed strategies for cancer biomarker discovery, validation, and translation. These strategies and specific examples of our studies are listed below.

- **Select the right technologies: Protein array and/or mass spectrometry**. Zhang H & Chan DW. Cancer biomarker discovery in plasma using a tissue-targeted proteomic approach. Cancer Epidemiol Biomarkers Prev (2007) 16:1-3.
- **Use well characterized clinical specimens: Pathology**. Rai AJ….Chan DW. HUPO Plasma Proteome Project specimen collection and handling: Towards the standardization of parameters for plasma proteome samples. Proteomics (2005)5:3262-3277.
- **Develop bioinformatics tools for data analysis and multiplexing of biomarkers: Engineering.** Zhang Z et al. Combining multiple serum tumor markers improves detection of stage I epithelial ovarian cancer. Gynecol Oncol (2007)107: 526-31.
- **Design multi-center case control study with extensive clinical validation to minimize the impact of possible confounding variables: Statistics.** Zhang Z & Chan DW. Cancer Proteomics: In Pursuit of "True" Biomarker Discovery. Cancer Epidemiol Biomarkers Prev (2005)14:2283-2286.
- **Discover and identify biomarkers with biological (clinical) significance: Cancer Biology.** Koopmann J et al. Serum macrophage inhibitory cytokine 1 as a marker of pancreatic and other periampullary cancers. Clin Cancer Research (2004)10:2386-2392.
- **Perform analytical validation to achieve high accuracy, obtain long-term consistent results, and achieve interchangeable results: Clinical Chemistry.** Meany DL and Chan DW. Comparability of tumor marker immunoassays: still an important issue for clinical diagnostics? Clin Chem Lab Med (2008)46:575–576.
- **Perform clinical validation to achieve high accuracy, sensitivity, specificity and diagnostic performance (ROC analysis): Clinical Chemistry.** Sokoll LJ…Chan DW. (-2) Proenzyme PSA for prostate cancer detection: An NCI-EDRN validation study. J Urology (2008)180: 539-543. Current status: clinical trial being conducted by a public/private partnership of EDRN/Beckman Inc with intent for FDA approval as a clinical diagnostic.
- **Translate biomarkers into multiplexed clinical diagnostics: Clinical Practice.** Zhang Z…Chan DW. Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer. Cancer Research (2004) 64: 5882-5890. Current status: Licensed to Vermillion, Inc., a clinical diagnostic has been developed and submitted to the FDA for approval.

Strategies have been implemented in our laboratory for the discovery and validation of proteomic biomarkers with the goal of translating these markers into clinical practice for the early detection of cancer. The above publications and FDA filings illustrate our capabilities and successes in translating cancer biomarkers from the bench to the bedside.

Keywords: biomarker, proteomics, cancer detection

085 Molecular Fingerprints of Cancer in Histologically-Normal Breast Tissues

Dung-Tsa Chen, Aejaz Nasir, Aedin Culhane, Chinnambally Venkataramu ,William Fulp, Renee Rubio, Tao Wang, Deepak Agrawal, Mike Gruidl, Gregory Bloom, Tove Anderson, Joe White, John Quackenbush, **Timothy Yeatman**

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Purpose: Historical data have reported up to a 40% local recurrence rate in the ipsilateral breast after lumpectomy without radiotherapy, which can be reduced to 10% with radiotherapy. These data demonstrate the potential for the histologically-normal breast to harbor pre-malignant changes at the molecular level.

Materials and Methods: From a total of 90 breast cancer patients, we collected a set of 143 histologically-normal breast tissues derived from patients harboring breast cancer who underwent curative mastectomy, which were completely-free of any other breast lesions, as well as a set of 42 histologically-invasive ductal carcinomas (IDC) of various histologic grades. All samples were assessed for global gene expression differences using microarray analysis.

Five external, independent data sets were collected to evaluate the malignancy-risk signature.

Results: Here we report that the analysis of 143 completely histologically-normal breast tissues resulted in the identification of a “malignancy risk” gene signature that may serve as a marker of subsequent risk of breast cancer development. Evaluation of the malignancy-risk signature using five independent data sets indicated that the signature had multiple properties, including potential to identify cancer risk, disease progression, and metastasis. Moreover, unlike invasive cancers expressing genes linked to proliferation and adhesion, the malignancy-risk signature, derived from normal tissues, showed a marked enrichment for genes with proliferative function.

Conclusion: These results suggest a critical role for the malignancy risk signature genes in the earliest stages of breast cancer development.

Keywords: breast tissue, metastasis, gene signatures

086 Molecular Profiling of Breast Cancer

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Major challenges in achieving a personalized approach to directing breast cancer therapy include extracting appropriate information to accurately define each *individual* patient's prognosis (outcome independent of treatment) and to predict which therapeutic regimens will provide the best clinical outcome. Effectively addressing these challenges is confounded by the highly heterogeneous nature of the disease; breast cancers with similar histopathological appearance can follow quite distinct clinical courses and show different responses to therapy. Consequently, many women with small, node-negative breast cancers are essentially overtreated *e.g.,* most Stage I breast cancers are treated with both local and systemic therapies but ~80% are effectively cured with local interventions alone. Separating these patients from the ~20% who recur, irrespective of their treatment, remains problematic (prognostic goal). Consequently, the development of novel methods that can more accurately predict for a nonrecurrent vs. recurrent phenotype is a major priority.

Specifically, we hypothesize that differences in the gene expression profiles of tumors determine outcome (recurrence) in patients with nonmetastatic disease. We also hypothesize that computational bioinformatics can discover these differences and use this knowledge to build classifiers that predict each individual patient's prognosis (especially in Stage I disease). Our multidisciplinary teams are collecting molecular profiles and established prognostic factor data from breast cancer patients to build artificial intelligence-based classifiers and multivariate models that accurately predict those patients with nonmetastatic disease (especially Stage I) who will/will not recur.

To achieve this goal, a major focus of this bioengineering research partnership R01 award is to develop powerful new tools and approaches to optimize the collection, quality, normalization, visualization, and analysis of gene expression microarray data. These tools are being collected into a caBIG compatible bioinformatics suite for data modeling and analysis. The clinical and microarray data are being collected in both a retrospective study and in a large prospective analysis of early stage breast cancers.

For the clinical component, we have currently acquired over 400 retrospective cases (n=418 specimens) and accrued 315 prospective breast cancer cases (n=1,170 specimens). To date, we have focused on the retrospective study, as we are collecting clinical outcomes data from the prospective study and accrual continues. We have completed initial analyses of a subset of the retrospective data in ER+ breast cancers and compared initial predictors of clinical outcomes.

For the bioengineering component, we have created, tested, and validated new methods for tissue heterogeneity correction (an *in silico* tissue microdissection method), normalization (supervised, cross-phenotype, linear and non-linear methods), data visualization (suite of tools, *i.e.,* caBIG VISDA), multitask gene selection (several new univariate and multivariate methods), classification and prediction (optimized multilayer perceptrons and novel applications of support vector machines). While not a component of the initial study, we have also begun to develop powerful new tools to extract molecular signaling pathways based on several approaches including knowledge-guided multiscale independent component analysis, differential dependency network modeling, and particle swarm optimization methods. In all cases, our tools are validated by statistically comparing performance against the appropriate tools most commonly used in the research community.

Keywords: microarrays, bioinformatics, prognosis

087 Inherited Susceptibility to Tumor Progression and Metastasis

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Tumor metastasis is one of the most important clinical aspects of neoplastic disease since patient mortality is frequently due to disseminated, rather than primary tumors. However, in spite of the importance of this process and the significant investment of resources to investigate it, great gaps remain in our understanding of the mechanisms of metastasis. To gain a better understanding of metastasis, our laboratory has investigated how an individual's inheritance influences the propensity of a tumor to disseminate. Using a highly metastatic transgenic mouse mammary tumor model we demonstrated the presence of polymorphic loci in the genome that had a significant impact on the ability of tumors to colonize the lung. Subsequently, using a variety of methodologies, we have identified the first of these polymorphic genes, *Sipa1*, and demonstrated that modest changes in gene expression or function strongly impact metastatic efficiency. More recently, we have been investigating the origins of the recently described tumor-derived metastasis-predictive gene expression signatures. Exploration of our mouse model has demonstrated that many of the genes comprising these signatures are differentially expressed in normal tissue as well as tumor tissue, suggesting that constitutional polymorphisms, rather than just somatic events, are a major factor influencing the predictive expression patterns. Genetic mapping of the polymorphisms controlling the expression pattern of the metastasis predictive signature genes has revealed loci that co-localize with our previously mapped metastasis efficiency modifier loci. Further analysis of these loci has identified a number of polymorphic genes, that when ectopically expressed in a highly metastatic mammary tumor cell line, alter both the expression patterns of the endogenous metastasis-predictive signature genes and progression and metastasis after subcutaneous implantation in mice, and that these signatures predict outcome in multiple independent human breast cancer expression datasets. These results suggest that inherited polymorphism plays a significant role in the establishment of metastatic susceptibility and may provide an additional tool for clinical diagnostic and prognostic evaluation of patient risk and therapeutic choice.

Keywords: genetics, metastasis, susceptibility

088 Gene Signature of Cancer Stem Cells is Manifested Within an Intrinsic Subgroup of Breast Cancers With Mesenchymal Properties

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Background: Breast cancer stem cells characterized by $CD44^{+}/CD24^{-/low}$ may be resistant to therapy and responsible for relapse. Mammospheres (MSs) can be propogated as an in vitro surrogate assay for increased self-renewal. We set out to define a "signature" expression pattern ssociated with $CD44^{+}/CD24^{-/low}$, mammosphere-forming cells.

Methods: Breast cancer biopsies (n=19) were digested, stained with CD24, CD44, and lineage antibodies, and analyzed by flow cytometry. A portion of the unsorted cells were plated under serum-free conditions to form MSs (n=16). Gene expression, using the Affymetrix U133 GeneChip platform, of cancer cells bearing $CD44^{+}/CD24^{-/low}$ *vs.* other sorted cells, and between cancer MS *vs*. the primary invasive cancers were analyzed. Gene expression from two trials (neoadjuvant letrozole N=18, and docetaxel, N=12) were used as validation studies.

Results: In the flow-sorted $CD44^{+}/CD24^{-/low}$ *vs.* other cells, 1,424 named genes were elevated ($p<0.01$, fold change>1.5, FDR=0.20). The comparison between MSs *vs.* primary cancers yielded 1,890 elevated genes (FDR=0.25). Between the two sets, 380 genes were in common, a highly significant overlap (p=1E-5, one-sided Fisher's exact). This overlap was ~40% greater than what would be expected (n=110) if the two sets had no biological relevance. Differential pathways included genes in PI3K/AKT signaling (PI3K3R3, ErbB3, FGFR2, PRLR), and the Notch pathway—a known regulator of normal and malignant stem/progenitor cells (Jagged-2, MAML2, Deltex, HES1). This signature was found exclusively activated in tumors of the recently identified "claudin-low" subtype, characterized by overexpression of many mesenchymal genes. Both signatures were validated in two independent data sets comparing the expression profiles of paired breast cancer core biopsies before vs. after letrozole or docetaxel chemotherapy.

Conclusion: The mesenchymal association provides a possible explanation for the intrinsic resistance of breast cancer stem cells to therapy.

Keywords: cancer stem cells, epithelial mesenchymal transition, therapy resistance

089 Development of the "PAM50" Intrinsic Subtype Assay: Potential as a Prognostic Assay for Patients With Early Stage Breast Cancer and as a Predictive Assay for Patients Undergoing Neoadjuvant Aromatase Inhibitor Therapy for ER+ Disease

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Background: We have shown by microarray and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) that breast tumors can be reproducibly classified into five distinct groups based on the expression profile of a "minimal" intrinsic gene list henceforth called the PAM50. The PAM50 identifies two ER+ subgroups - Luminal A (LumA) and Luminal B (LumB), two ER- subgroups - HER2-enriched (HER2) and Basal-like, and a final category Normal-like which has an expression pattern that most closely resembles non-malignant breast tissue. This biologic classification has been shown to be an independent predictor of disease free survival when considering standard clinical parameters. However, risk predictors based on these gene expression profiles have been difficult to clinically implement. We sought an objective method to predict subtypes of breast cancer and generate a continuous risk score based on a biological subtype predictor that can be performed on archived tissue blocks using real-time qRT-PCR.

Methods: Microarray and real-time qRT-PCR data from 189 samples, procured as fresh frozen and formalin-fixed paraffin-embedded tissues, were used to statistically select prototypes for the biological subtypes of breast cancer. Classification algorithms were developed using four independent breast microarray studies together comprising 1244 cases. From these data, a risk of recurrence (ROR) predictor was developed based on distance to the PAM50 subtype centroids. In addition, a proliferation score was generated from the expression of 11 tightly correlated, cell cycle-regulated genes in the PAM50 to create a tailored ROR score for response to neoadjuvant aromatase inhibitor therapy. A linear relationship between the ROR score and relapse risk was identified across the cohorts.

Results: The biological subtypes predicted on the combined microarray test sets showed prognostic significance in all stages of disease (1244 subjects; p=7.1e-14), node negative disease with no adjuvant systemic therapy (733 subjects; p=6.2e-7), and in patients treated with endocrine therapy (404 subjects; p=1.3e-3). The proliferation weighted ROR model applied to tumor expression profiles generated from baseline biopsy, one month neoadjuvant treatment, and subsequent tumor excision at surgery showed marked changes with aromatase inhibitor treatment, and robustly identified a group of resistant tumors on the basis of a persistently high risk of relapse score one month after treatment. These resistant tumors were associated with a poor clinical response rate, poor pathologic response in the surgical specimen, and high relapse rate in follow-up.

Conclusion: A qRT-PCR panel of 50 discriminator genes, applicable to standard pathology blocks from biopsy and excision specimens, robustly identifies the intrinsic biological subtypes of breast cancer and predicts response to aromatase inhibitor therapy as well as overall prognosis in independent series.

Keywords: quantitative RT-PCR, bioclassifier, aromatase inhibitor

090 Novel Approaches to Cancer Biomarker Discovery Using Proteomics

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Although our understanding of the molecular pathogenesis of common types of cancers has improved considerably, the development of effective strategies for early cancer diagnosis have lagged behind. We have implemented strategies for in-depth quantitative proteomics of the plasma proteome to identify markers for early cancer detection. The strategy consists of analysis of circulating proteins and autoantibodies to tumor antigens in specimens obtained at the time of diagnosis and at a pre-clinical stage, prior to the diagnosis of cancer. In parallel, we have utilized mouse models of cancer that represent an efficient means for uncovering diagnostic markers because of the ability to engineer mice that harbor genetic alterations known to be associated with tumors in humans, and because of the limited heterogeneity among mice bred under uniform conditions and the ability to sample blood in a standardized manner, at defined stages of tumor development. Findings from studies of epithelial cancers, notably lung and pancreatic cancer, that progressed from discovery to blinded validation in pre-diagnostic sera will be presented that demonstrate the power of current proteomics strategies to yield markers for early cancer detection.

Keywords: early detection, biomarker, proteomics

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091 Integrated DNA Methylation Analysis of Glioblastoma: The TCGA Consortium

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Aberrant hypermethylation of CpG dinucleotides located in CpG islands within the promoters of key cancer genes is an epigenetic abnormality associated with heritable transcriptional gene silencing and inactivation in cancer. Studies of major cancer types suggest that any individual patient's tumor may harbor upwards of 300 DNA hypermethylated genes. We used Illumina GoldenGate with a custom GBM array to monitor methylation at a single CpG dinucleotide in the CpG islands of 1,498 gene promoters identified from epigenetic reactivation studies of glioblastoma cell lines and the commercial Illumina Cancer Panel I array, containing an additional 807 non-overlapping gene promoters, to query 215 primary GBM samples from 195 patients. An algorithm was derived to identify genes specifically DNA hypermethylated in GBM versus normal brain which identifies genes satisfying the criteria: (a) unmethylated in negative control DNA; (b) methylated in positive control DNA; (c) unmethylated in all available normal brain samples; (d) partially methylated in at least 10% of primary tumors; (e) fully methylated in at least 25% of primary tumors and (f) a Spearman correlation >0.3 between gene expression and DNA methylation values.

We have identified approximately 300 "cancer-specific" hypermethylated GBM genes using this approach. Heat map analyses identified at least three gene and tumor clusters that may be of biologic and clinical interest. These clusters were maintained by refining to a final list of 45 genes, through integration with gene expression platforms in TCGA, with each gene having increasing methylation with decreasing gene expression. These genes include critical pathways involving the retinoid signal transduction pathway (RBP1, FABP5), which is key to proper neural differentiation, and tyrosine kinases (EPHA2, EPHB1) which are essential for normal brain development. Integrated approaches with regions of genetic copy number alterations identify O6-MGMT, and the chemonkine receptor CXCL12 as frequent targets of both epigenetic and genetic inactivation. Methylation of CXCL12 is associated with reduced survival.

Integration of the methylation data with sequencing efforts reveals the importance of genetic and epigenetic interactions. A list of 601 genes selected to include known tumor suppressor genes and oncogenes as well as genes related to glioma pathogenesis were sequenced in 91 matched tumor-normal pairs. Included in our study was methylation of the MGMT gene, which encodes a DNA repair protein that removes alkyl groups from guanine residues, thereby preventing DNA cross-links and cell death, and whose promoter methylation has been correlated with GBM sensitivity to alkylating agents. MGMT was methylated in 20 of 94 the sequenced tumors, while MLH3, a mismatch repair gene not previously known to be altered by DNA methylation, was methylated in six of the 94 tumors. Additional mutations were observed in the mismatch repair genes MLH1, MSH2, and MSH6. The twenty tumors with MGMT methylation harbored 206 mutations among the 601 sequenced genes (mean 10.3 per tumor), compared to 248 mutations in 74 tumors without MGMT methylation (mean 3.5 per tumor). Loss of MGMT is associated with G to A mutations (or C to T on the opposite DNA strand). Consistent with this, tumors with MGMT methylation had 79% of mutations being Gto A changes and only 8% at CpG dinucleotides (resulting from spontaneous deamination of 5-methylcytosine), while the frequencies in tumors without MGMT methylation were 48% and 52%, respectively. These differences are statistically significant (G to A mutations, p=3x10-12 and CpG mutations, p=2x10-16). Thus, both DNA repair gene mutations and epigenetic alterations may affect the overall frequency and pattern of mutations in GBM tumors.

These findings from TCGA may allow further classifications of GBM for response to the primary therapy of alkylating agents combined with radiation therapy, and new pathways such as Ephrin and retinoid signaling, and inflammatory chemonkine signaling as targets for therapeutic intervention.

Keywords: glioblastoma, genomic analysis, epigenetics

092 Prospective Identification of Reverse Phase Proteomic Signatures of Estrogen Resistance in High-Risk Premenopausal Women

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AKT/protein kinase B is a family of protein kinases that regulate proliferation, apoptosis, and damage-response-signaling. High AKT activity and phosphorylation at Ser473 is associated with resistance to Tam chemotherapy. Normal mammary gland homeostasis involves the coordinated regulation of cellular signaling networks. Our *in vitro* and initial studies in high-risk women provided evidence that disregulation of AKT-signaling occurs during initiation of estrogen-resistant breast cancer. In this study, we used Reverse Phase Proteomic Microarray analysis (RPPM) to prospectively test for disregulation of phosphorylation signaling from premenopausal women in our established high-risk cohort.

Random Periareolar Fine Needle Aspiration (RPFNA) is a research procedure developed to identify 1) short-term breast cancer risk and 2) response to breast cancer prevention. Over the past 4 years performed serial RPFNA on a cohort of 257 high-risk women. Using RPPM we identified an initial proteomic signature of estrogen-resistance and short-term progression in high-risk women with mammary atypia who took tamoxifen for 12 months and either had 1) persistent atypia or 2) disappearance of atypia. Guided by these initial data, RPPM proteomic profiling has focused on signaling pathways regulated AKT, PTEN, Bcl-2, EGFR, PI3K, mTor, and E-cadherin.

Computational analysis of protein patterns was performed using supervised and unsupervised methods as previously published [Petricoin et al. Cancer Research, 2007]. Supervised techniques included ANOVAQ, t-tests, and Wilcoxon rank scores. Significant single phospho-protein associations ($p<0.05$) were observed with AKT-pSer473, p70S6 kinasepThr412, 4EBP1, IKB-pSer32-36, and E-cadherin. Full analysis is on-going.

Conclusion: Our data provide evidence that AKT/mTor-signaling is disregulated in high-risk women who fail tamoxifen prevention. Our studies provide a mechanism to rapidly identify women who fail tamoxifen prevention and provide insight into the biology of estrogen-resistant breast cancer initiation.

Significance: Currently we lack agents to ***prevent*** estrogen-resistant breast cancer. Targeted agents are undergoing clinical testing for ***treatment*** of estrogen breast cancer. Low toxicity targeted agents represent an important future opportunity to prevent estrogen-resistant breast cancer in women who fail Tam.

However, In order to most effectively use targeted agents for prevention of estrogen-resistant breast cancer we need to first develop a mechanism to identify sensitivity of the targeted tissue. Without predictive biomarkers Phase I testing of targeted agents for prevention of estrogen-resistant breast cancer will be too risky, expensive, and will not select women with the highest likelihood of response. Our proteomic signatures provide a mechanism to identify signaling pathways that are intact in Tam-resistant breast tissue, guide selection of a targeted agent that has the highest likelihood of success, and then track response to the agent.

Keywords: breast cancer risk assessment, proteomics, AKT

093 Development and Standardization of Liquid Chromatography-Tandem Mass Spectrometry Methods for Protein Biomarker Discovery and Verification in Tissues

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Research at Vanderbilt supported by the NCI Clinical Proteomic Technologies Assessment for Cancer (CPTAC) program addresses goals in 1) credentialing, 2) supporting tools and 3) creation of modality elements of the TRWG Biospecimen-Based Assessment Tool Pathway. Shotgun proteome analysis platforms based on multidimensional liquid chromatography-tandem mass spectrometry (LC-MS-MS) provide a powerful means to discover biomarker candidates in tissue specimens. We compared shotgun proteomics platforms by analyzing tryptic digests of whole cell and tissue proteomes using strong cation exchange (SCX) and isoelectric focusing (IEF) separations of peptides prior to LC-MS-MS analysis on a LTQ-Orbitrap hybrid instrument. IEF separations provided superior reproducibility and resolution for peptide fractionation. High reproducibility and efficient resolution of IEF peptide separations make the IEF platform well suited to biomarker discovery via shotgun proteomic analyses of tissue specimens. LC-MS-MS analyses with this platform have been done in collaboration with the other CPTAC centers through a Discovery working group series of interlab studies.

The IEF-based shotgun proteomics platform has been extended to analysis of formalin-fixed paraffin-embedded (FFPE) tissue, which is an important source of tissue for retrospective biomarker discovery and verification studies. Protein identification differences are not highly sensitive to preprocessing variables, such as fixation time and duration of storage (up to 10 years). We also have developed a data analysis pipeline for shotgun proteomics built around the Vanderbilt-developed, open source tools Scansifter, Myrimatch and IDPicker, and a new statistical tool for the comparison of shotgun proteomics datasets based on a modified Poisson regression model to facilitate comparisons by spectral counts of datasets with "missing" values, which is characteristic of shotgun proteomics datasets.

We developed a liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM-MS) method to quantify protein biomarker candidates in tissues without the use of antibodies or stable isotope labeled standards. MRM-derived signals for target peptides from proteins of interest are normalized to signals for peptides from endogenous tissue proteins. This approach enables rapid biomarker candidate screens and bridges biomarker discovery platforms and more costly stable isotope dilution LC-MRM-MS or immunochemical methods. In studies with all CPTAC centers, we have evaluated the performance characteristics of stable isotope dilution LC-MRM-MS for prototypical biomarkers proteins and peptides in plasma. (Supported by National Institutes of Health Grant 1U24CA126479.)

Keywords: proteomics, colon cancer, biomarkers

094 EDRN Biomarker Development Lab: Prostate and Bladder Cancer Genes

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Our approach is to use genomics for discovery and proteomics for detection of secreted proteins. We applied cell sorting to isolate cancer cells from primary tumors for analysis by Affymetrix GeneChips. Dataset analysis between cancer cell transcriptomes and those of normal cell types produced a cohort of differentially expressed genes. Candidates were those with increased cancer expression that encode secreted or extracellular proteins. Tumor-associated stromal fibromuscular cells, which showed differential gene expression from stromal cells of normal tissue, provided another source of biomarkers.

$CD26^+$ cancer cells were sorted from prostate cancer case 05-179 (T2cN0Mx, Gleason 3+3, PSA=10.6, 4.7 cc tumor volume, 44y patient). An array data analysis tool, HTself, was used to identify 121 up-regulated and 86 down-regulated genes by ≥8-fold in these cancer cells *vs*. $CD26^+$ luminal cells, the normal counterpart. Genes encoding secreted proteins include AGR2, BCMP11, CEACAM5 and CRISP3. Increased cancer expression was verified by Western blotting of tissue proteins.

CD90 immunostaining showed that prostate tumor-associated (CP) stromal cells were readily distinguishable from benign tissue (NP) stromal cells. The $CD90^+$ CP stromal cells constituted not more than 10 cell layers surrounding the cancer cells. Dataset comparison between $CD90^+$ CP and $CD49a^+$ NP stromal cells showed that certain prostate-specific (i.e., not in bladder) stromal genes were down-regulated in cancer. Increased expression of CD90 was detected by Western. The secreted SFRP4 is an up-regulated gene identified through dataset comparison.

Mass spectrometry-based proteomics was applied to measure proteins derived from cancer. By the glycopeptide-capture method, *N*-glycosylated CD90 was found to be 5-fold higher in CP than NP. By the ICAT method, CD90 was shown, like PSA, to be present in pre-op(erative) urine and not in post-op urine. By the MRM method using isotopically labeled signature peptides, a higher amount of CD90 was measured in pre-op *vs*. biopsy negative and non-cancer urine.

In addition to markers for cancer detection, those for disease stratification were identified. Dataset comparison was performed between $CD26^+/CD10^-$ cancer cells and $CD26^-/CD10^+$ LNCaP or $CD26^+/CD10^+$ C4-2 cells because a $CD10^+$ cancer cell type was found enriched in positive lymph nodes. Cell lines and xenografts developed from node metastasis are $CD10^+$. Because of its lower frequency, the $CD10^+$ cancer cell type cannot be readily isolated from CP. The analysis identified MAGE and GAGE genes, among others, being differentially expressed. Western blotting was used to confirm expression in node specimens.

Markers associated with stem cells are likely associated with cancer stem or tumor promoting cells. Stem cells respond to stromal cell signaling by undergoing differentiation. Genes down-regulated in this process are candidate stem cell markers. We used an EC cell line and prostate stromal cells to identify these genes. One example found was cytoplasmic tumor protein TPD52/PrLZ, which was previously reported to be expressed in androgen independent prostate cancer. Only 5% of the down-regulated genes were predicted to encode secretory proteins (*vs*. 20% in differentiated EC cells). Among them, APOE and PROK2 have been reported to be associated with advanced cancer.

Keywords: cancer biomarkers, cell type-specific transcriptomes, secreted proteins

095 PEPPeR: A High-Throughput Platform for Pattern-Based Biomarker Discovery Using Fractionated Samples

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Quantitative proteomics using liquid chromatography coupled to mass spectrometry (LC-MS) holds considerable promise for elucidation of basic biology, and for clinical biomarker discovery. However, effectively harnessing the wealth of information in LC-MS data necessitates going beyond the traditional approach of analyzing only identified peptides. To address these challenges and facilitate robust and high throughput biomarker discovery, we have developed PEPPeR—A *P*latform for *E*xperimental *P*roteomic *P*atte*r*n *R*ecognition. PEPPeR uses high resolution and high mass accuracy LC-MS data from state-of-the-art mass spectrometers, and appropriately combines pattern-based (unidentified peptide peaks) and identity-based (peptides sequenced via MS/MS) information to generate peptide quantitation for biomarker discovery. Furthermore, PEPPeR is capable of computationally reassembling peptide fractions from multidimensional fractionation to facilitate data analysis at the sample level, in spite of imprecise fraction boundaries or other variations during fractionation.

The PEPPeR platform has been evaluated using a variety of protein mixtures, mitochondrial extracts with spiked in peptides, SCX fractionated yeast samples, nipple aspirate fluid from breast cancer / control patients, plasma from mouse models of lung adenocarcinoma, and multiple time point plasma samples from patients being treated for hypertrophic cardiomyopathy. With protein mixtures, we have shown that the platform can be used to quantify mass spectral features corresponding to peptides across 2-3 orders of magnitude (1 fmol to 300 fmol). In a model mimicking sample variability likely to be encountered during biomarker discovery, we were able to accurately quantify ratios between two samples ranging from 0.1 to 50-fold with an average absolute deviation of 15% from the known ratios, and an average inter-sample intensity CV of 17%. Using marker selection methods like the t-test, we estimate false positive and false negative differential marker identification rates of 11% and 16% respectively, based on a multiple hypothesis corrected p-value cutoff of 0.01. When comparing computationally reassembled fractions with the unfractionated sample, we obtain very comparable results, with almost identical median intensities in the two sets.

Putative biomarkers identified using PEPPeR can be efficiently subject to accurate mass inclusion scanning (AIMS) for quick identification and follow up.

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Keywords: biomarker discovery, pattern recognition, LCMS label-free quantitation

096 Proteomic Mapping of Endothelial Caveolae to Pump Radio-Antibodies Into Tumors for Specific Imaging and Therapy

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Cancer and other disease biomarkers and targets may provide key diagnostic, prognostic and therapeutic opportunities including clinical trial surrogates and screens for patient treatment assignment. Drugs, gene vectors, and nanoparticles may benefit greatly from improved in vivo delivery through homing to specific disease biomarkers. Yet in vivo barriers limit access to most disease targets including cancer. We have developed novel systems biology approaches that integrate nanotechnology-based subcellular fractionation, quantitative organellar & subtractive proteomics, bioinformatic interrogation, antibody generation, expression profiling, and various in vivo imaging modalities to quickly identify and validate target candidates for pre-clinical and clinical testing. Analysis of rodent and human tumor samples have been compared to focus on clinical meaningful targets and to understand model relevance to human disease. Tissue and tumor microenvironmental influences on endothelial cell expression are extensive. We have developed quantitative proteomic analysis using a new spectral intensity index to identify proteins specific to tumor vs. normal endothelium as well as concentrated in caveolae; many of which are confirmed by immuno-electron microscopy. Novel targets in caveolae enable antibodies to penetrate deep into solid tumors and single organs and were utilized to improve tissue-specific imaging and treatment. Our recent findings reveal that caveolae not only express tissue-specific proteins but also function to rapidly and actively pump specifically targeted antibodies and nanoparticles across the endothelial cell barrier and into the tissue interstitium. This targeted penetration of the antibody into the tissue (transcytosis) occurs within seconds to minutes in normal tissues and with in minutes to a few hours in various tumor models tested. Such pervasive access inside the tumor improves the efficacy of radioimmunotherapy in destroying both stromal and tumor cells and in treating a wide variety of solid tumors. The first antibody that we wish to test clinically recognizes annexin A1 which appears tumor-induced and –specific on the outside surface of endothelia in vivo based on proteomic imaging data already published (Oh et al., Nature, 2004). Various rodent tumors are imaged rapidly and specifically after intravenous injection of specific monoclonal antibodies. This radioimmunotherapy effectively destroys tumors in rodent models to increase survival and even apparently cure the disease. So far, we have tested breast, lung, ovarian, prostate, and liver tumors with similar success. We have antibodies that recognize this target in humans. A wide variety of human tumors express this novel accessible endothelial cell surface target in a pattern quite similar to the rodent models. We are testing different radionuclides to evaluate which one is most effective. Toxicology studies are ongoing. Our antibody appears useful in tumor-specific imaging as well as in treating a wide variety of solid tumors. This work represents a novel discovery, validation and delivery strategy that so far provides promising and unprecedented results. Testing in humans is now necessary to understand limitations and possibilities for clinical translation to imaging and treating human disease.

Keywords: vascular endothelium, radiommmuno-imaging, radiommmuno-therapy

097 Molecular Classification of Suspicious Thyroid Tumors

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Background: Although thyroid cancer is one of the fastest growing cancers in the United States, the incidence having doubled in the last decade (http://seer.cancer.gov), cytopathologists often experience difficulties in preoperatively diagnosing thyroid lesions as either benign or malignant. As a result, 20-25% of fine needle aspirates (FNA) are reported as indeterminate, and 10-15% as inadequate for diagnosis. Furthermore, knowing there are 37,000 new thyroid cancers diagnosed as well as 350,000 FNAs performed annually, by extrapolation, there are up to 140,000 patients a year who present in the United States with indeterminate thyroid neoplasms (http://www.cancer.org). Because the clinician and surgeon cannot determine malignancy pre- or intra-operatively, patients with indeterminate thyroid lesions on FNA cannot be optimally clinically managed. Therefore, additional diagnostic markers of malignancy are greatly needed.

Methods and Results: To address this problem we have analyzed by microarray analysis 94 thyroid tumors, 50 of which were benign (13 adenomatoid nodules, 13 follicular adenomas, 13 Hürthle cell adenomas and 11 lymphocytic thyroiditis nodules) and 44 malignant (13 papillary thyroid carcinomas, 13 follicular variant papillary thyroid carcinomas, 13 follicular carcinomas and 5 Hürthle cell carcinomas)[1]. Of 15,745 genes included in the analysis, 33 were significantly overexpressed and 42 underexpressed in malignant versus benign tumors. Prediction and cross-validation models suggested that the genetic analysis was 73% sensitive and 82% specific for the prediction of malignancy; the positive predictive value was 78%. Validation of 12 of the differentially expressed genes was carried out by real-time reverse transcription polymerase chain reaction; validated genes included *HMGA2*, *PLAG1*, *SPOCK1*, *CEACAM6*, *LRRK2*, *RAG2* and *AGTR1*.

Future Plans: We have begun to examine protein expression of the above-listed genes in thyroid tumors and thyroid FNA samples by immunohistochemistry and immunocytochemisrty, respectively and have documented differentiating expression levels between benign and malignant tumors for several of these markers. We anticipate developing a diagnostic panel to be useful in the differential diagnosis of indeterminate or suspicious thyroid nodules and, by so doing, help solve a clinical problem that 100,000 patients in the United States face annually.

Reference: 1. Prasad NB, S.H., Tufano RP, Dackiw APB, Marohn MR, Califano JA, Wang Y, Westra WH, Clark CP, Umbricht CB, Libutti SK, Zeiger, MA, Identification of Genes Differentially Expressed in Benign Versus Malignant Thyroid Tumors. Clin Cancer Res, 2008. 14(11): p. 3327-3337.

Keywords: thyroid, FNA, diagnosis

098 Preservation of Sample Integrity in Biobanked Samples Using a Frozen Sample Aliquotter

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Biological repositories represent valuable resources for basic and translational research and there are many large repositories, funded with government and private funds, and an even greater number of small and medium repositories. A key objective of these repositories is to make biological samples available to support and enable research on biomarkers, nutrition, functional genomics and many other avenues of research. It is imperative that the quality of the biological specimens be maintained or the research conducted using them will be compromised. A critical problem faced by repositories is the degradation of the RNA and some proteins in the samples due to freeze thaw cycling.

We have developed an automated instrument that extracts 100µl aliquots of serum from a 1.8ml cryovial without thawing the serum. The technology is also applicable to a variety of other frozen specimens, including: whole blood, cells, compounds in DMSO and urine.

We have demonstrated proof of principle for this technology in the areas of volumetric accuracy and repeatability, the ability to clean the needle between samples to prevent cross contamination, the ability to maintain the specimens below -70C and prevent the buildup of frost on the sample surface for several hours, automatically recognize previously cored regions of the tube, and the ability to maintain the biochemical composition of the sample. We are currently working on the next generation of the prototype that will be deployed in the biobank at Brown University for extensive testing.

We have transitioned from the R21 work at Harvard Medical School to a startup company, CryoXtract Instruments, LLC who will develop products based on this technology to support translational research by improving the fidelity of the specimens provided by repositories and increasing their productivity in delivering those specimens through automation

Keywords: biorepository, biomarkers, automation

099 A New Platform of Much Higher Throughput Proteomics for Biomarker Discovery and Validation

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Current proteomics analysis rates, costs, etc. are broadly inadequate for characterizing significantly large numbers of samples required for biomarker discovery and/or validation. We are presently developing a new platform that will provide greatly improved measurement throughput, sensitivity, robustness, and quantitative capability for cancer biomarker discovery, verification, and pre-clinical validation. A major analytical challenge we are addressing in this work is to achieve higher analysis throughput with a concurrent increase in sensitivity and dynamic range of measurements to allow detection and quantitation of lower abundance peptides and proteins from biological fluids.

The new proteomics platform encompasses fast capillary LC separation coupled to an ion mobility spectrometer (IMS) which is interfaced to a time-of-flight mass spectrometer (TOFMS). To increase instrument sensitivity, we have developed a novel IMS multiplexing approach. The total duration of initial proteomics analyses is ~15 min. The initial evaluation of LC-IMS-TOFMS system was performed using human blood plasma samples depleted of the 12 most abundant proteins spiked with peptides of known concentration, and showed a significant improvement in the sensitivity of measurements compared to conventional commercial platforms, in addition to much higher throughput. (Related instrumental advances are also being implemented in conjunction with a triple quadrupole MS for MRM based directed validation of selected candidate biomarkers with even greater sensitivity.)

We anticipate that the development of the new platform will enable the throughput needed for much more effective biomarker discovery efforts based upon the much greater numbers of samples that can be studied, and the better accounting of biological variation.

Keywords: mass spectrometry, high throughput, biomarker discovery

100 Discovery and Validation of Cancer Biomarkers Using Integrated Technology Platforms

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We are developing and applying a comprehensive set of technologies designed to accelerate the rate of discovery and validation of cancer biomarkers. For discovery, we are using the accurate mass tag (AMT) proteomics approach. For pre-clinical validation, we are using a cell-free expression system to generate antigens, a combination of traditional antibody production and yeast display of single-chain antibodies (scFv), and a sandwich ELISA microarray platform. This ELISA microarray platform is designed for the high-throughput, quantitative analysis of biomarker panels.

We conducted a proteomics analysis of 80 plasma samples from women with early-stage ductal or lobular cancer, or controls that were matched based on age, menopausal status and body mass index. In order to increase confidence in the resulting protein hits, our proteomics data were compared with a meta-analysis of published microarray gene expression data. The combined data analysis identified a number of proteins that are significantly altered in both the blood and tissue of breast cancer patients. Many of these proteins (e.g., IGFBP-3 and alpha-1-antitrypsin) are established biomarkers of breast cancer. The novel candidate biomarkers are enriched in secreted proteins, type I and II membrane proteins, and proteins we previously identified in nipple aspirate fluid, which is a concentrated source of proteins secreted by the breast.

We evaluated the cell-free protein expression system for generating 12 proteins that are candidate biomarkers for prostate cancer. These proteins did not express in E. coli or were expressed as insoluble inclusion bodies. In the cell-free system, all 12 proteins were expressed as soluble GST-tagged proteins.

We generated scFv antibodies against prostate specific antigen (PSA) using yeast display. We identified two scFv that worked as a sandwich pair and that had Kd values of ~1 nM. In the ELISA microarray platform, this scFv pair had a lower limit of detection of ~5 pg/ml. The scFv reagents also had low non-specific protein binding that was similar to IgGs. Comparison of PSA levels in human plasma using the microarray scFv and a commercial 96-well immunoassay gave data that were strongly correlated (R^2=0.95).

For the ELISA microarray, we have developed a prototype chip using commercial reagents for 24 candidate biomarkers in human blood plasma. This chip was used to evaluate plasma from women with either ER+/Her2- or Her2+/ER- breast cancer, and age-matched controls. This analysis identified five circulating proteins that were statistically different in at least one of breast cancer subtypes. We are currently developing our next-generation ELISA microarray chip to validate candidate biomarkers identified from the proteomics analysis and other sources.

Keywords: biomarkers, breast cancer, ELISA microarray

101 Risk Assessment for Prostate Cancer Detection

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University of Texas Health Sciences Center

Currently, 75% of men over age 50 have undergone PSA testing for early diagnosis of prostate cancer. PSA has been used along with digital rectal examination (DRE) as dichotomous tests, with biopsy recommended for PSA > 4.0 ng/mL or abnormal DRE. We examined in the Prostate Cancer Prevention Trial (PCPT) the individual contribution of race/ethnicity, PSA, DRE, age, family history of prostate cancer, PSA kinetics, and history of a prior negative prostate biopsy in 5519 men in whom all had undergone prostate biopsy at a range of PSA values.

All variables except for PSA kinetics independently predicting risk of prostate cancer and have been incorporated into an on-line risk calculator (found at: http://deb.uthscsa.edu/URORiskCalc/Pages/uroriskcalc.jsp). This calculator has been validated in three additional cohorts (two national and one regional with a high concentration of Hispanic men). The calculator's results demonstrates that PSA and DRE cannot be used independently as dichotomous prompts for prostate biopsy as doing so may overdetect low risk disease in young men and may delay detection of aggressive cancer in some men until cure is no longer possible.

Through Bayesian methodologies, we have now updated the calculator to include other markers, the first of which is PCA3. Additional markers planned through the EDRN include proPSA as well as BMI.

Keywords: screening, prostate cancer, PSA

102 Tumor Microenvironment Based Prediction in Prostate Cancer

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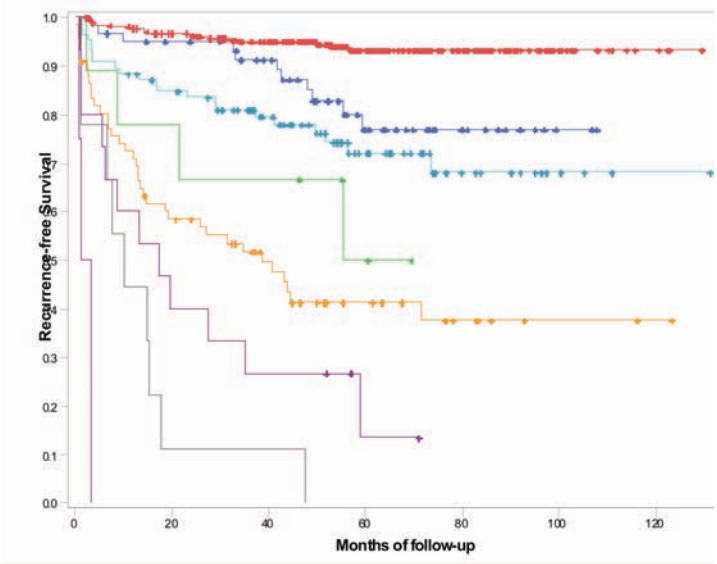
Extensive scientific literature data points to reciprocal interactions between prostate stromal cells and prostate cancer (PCa) cells. We have defined reactive stroma in prostate cancer both functionally and biologically. To investigate if these intratumoral reactive stromal cells in human PCa are predictive of survival, reactive tumor stroma volume was quantitated on TMAs. The relative volume of intratumor stroma (5% stroma=grade 0; 5 to 15%=1;15% to 50%=2; > 50%=3) was quantitated and analyzed. Tumors with no stroma (RSG 0) and large amounts of reactive stroma (RSG 3) were associated with adverse prognosis, independent of other biomarkers used today. Subsequently reactive stroma was then analyzed on H&E sections of the radical prostatectomies specimens (869 patients). The percentage of the tumor composed of RSG 0/3 was quantified and analyzed. Results show is an independent predictor of biochemical survival even as a continuous variable (p=0.000, HR=1.124). Finally 224 cases of prostatic needle biopsies diagnosed as prostatic carcinoma from 1988 to 1998 were used to quantify stroma on H&E stains. By Cox proportional hazard analysis, RSG was an independent predictor of recurrence (Hazard Ratio, 1.953; P=0.0174) in the overall population. In the Gleason 7 subset it displaces 3+4 vs. 4+3 from the model.

To identify interactions between stromal elements in the Prediction of PCa we tested a multivariate model with interaction that showed how PNI diameter adds predictive information to RSG and whether these can be used coordinately for better prediction even in the presence of all other currently used clinico-pathologic parameters. This is proof of concept that stromal microenvironment elements not only have biologic interactions, as demonstrated biologically between nerves and stroma, but that this biologic interaction also translates into the predictive realm (p=0.0062). PNI diameter adds predictive information to RSG and can be used in combination, particularly in prostatectomies.



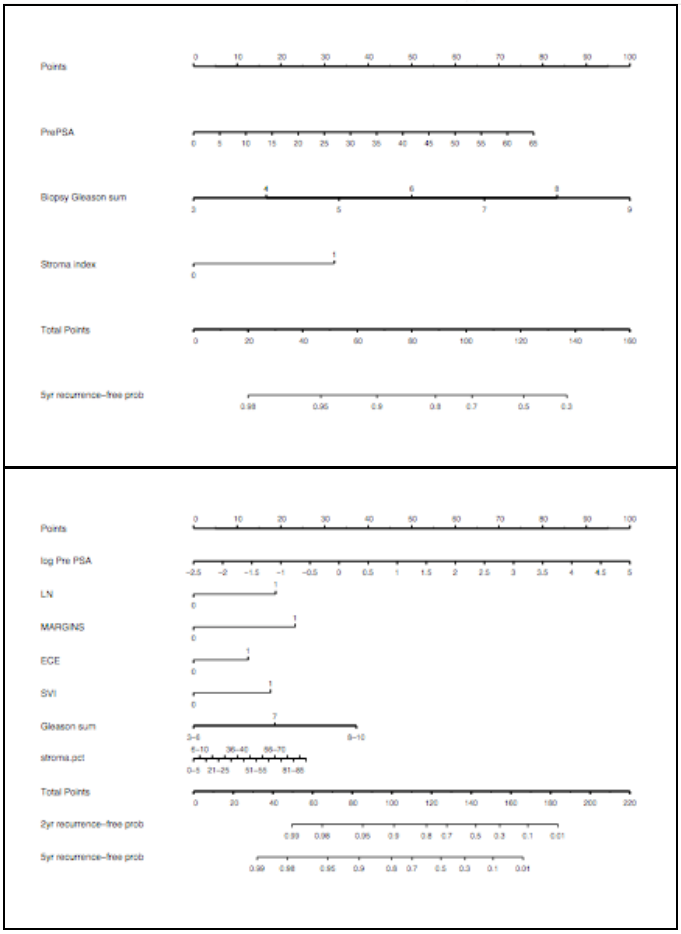


Figure 1A: Survival curves of TMEN prediction combination

Figure 1B&C: Pre and post operative nomograms for PCa that include TMEN prediction

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This is demonstrated in survival curves that combine the predictive power of tumor microenvironment elements in Pca (Fig 1A).

Based on the biochemical recurrence-free survival models described above, we developed a preoperative and postoperative nomogram and an electronic calculator for the risk of biochemical recurrence, based on the usual clinical/pathological parameters such as Preoperative PSA, Lymph node status, Margins, ECE, SVI, and Gleason Grade, including RSG. We have produced 2 nomogram models demonstrating that RSG grading provides significant and different information that carries similar weight to Gleason grading, both in the pre (Fig 1B) and postoperative (Fig. 1C) scenarios. Input of the required values into a user-friendly program screen displays a probability of staying biochemical recurrence free at 2 and 5-year marks. The calculator is presented in the form of an Access program.

This is the first study to demonstrate that non-epithelial reactive stroma elements in prostate cancer tumors can be used as prognostic indicators. This data also adds to the concept that tumors are not purely epithelial and the epigenetic tumoral and host events must be considered an important biological component of the cancer.

Keywords: prostate cancer, reactive stroma, perineural invasion

103 Molecular Effects of Nutritional Supplements on the Prostate Microenvironment

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(Drs. Haqq and Carroll share senior authorship on this study)

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Background: Observational epidemiologic and laboratory data have indicated that tomatoes (rich in the antioxidant lycopene) and fish oil (rich in omega-3 fatty acids) may deter prostate cancer incidence. There has been extremely limited data focused on the possible roles of these nutrients after diagnosis. Examining changes in the prostate tissue from men on active surveillance regimens provides a unique opportunity to study the natural progression of prostate cancer *in vivo*.

Design and Methods: We conducted a randomized blinded placebo-controlled clinical trial of tomato extract and fish oil in men with low-burden prostate cancer, who elected active surveillance as their primary management strategy. Men received a placebo, 3g of fish oil supplement, or a tomato extract supplement containing 30 mg lycopene for 3 months. Our main outcome was change in expression in genes of interest (e.g. in the *IGF-I* and *COX2* pathways) based on biopsies taken pre- and post-intervention, using cDNA expression array analyses.

Results: Ninety-five men were randomized in this study, but 11 became ineligible due to non-compliance, disease progression, or voluntary withdrawal. 84 participants who completed the intervention were eligible for final analyses. Analysis of the baseline data indicates the similarity in gene expression profiles across treatment arms. We remain blinded and statistical analyses are ongoing for our primary outcomes.

Conclusions: At this stage, this trial indicates the feasibility of enrolling and conducting translational nutritional intervention research in men electing active surveillance for prostate cancer. Analyses of our primary aims are anticipated to be ready by the end of summer 2008.

Keywords: prostate cancer, fish oil, tomato

104 Prostate Cancer Metabolomics

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Currently, genomics evaluates thousands of genes simultaneously and correlates their genomic profiles, instead of the expressions of single genes, with a particular disease condition. In parallel, all the metabolite processes (pathways), or metabolomics, in a biological system are also interconnected and should be evaluated simultaneously. The alterations of the overall metabolomic profiles are more sensitive and specific to a particular physiological and/or pathological condition than the changes in any single metabolite. Disease metabolomic profile(s) can be defined ex vivo by principle component analysis of MRS measurements from diseased specimens. In vivo disease detection may then be achieved by constructing the defined profiles with metabolite parameters measured by in vivo MRS (CSI), and mapping the resulting values for all the voxels on an anatomic image to detect and reflect degrees of disease involvements in the anatomy.

Five prostates from cancer patient prostatectomies were removed, kept on ice, and analyzed within two hours for this study. Two- and three-dimensional CSI analyses were conducted at room temperature, on a 7.0 T scanner. Afterwards specimens were fixed for histological evaluation. Metabolite intensities (n=36) from CSI data for each voxel were processed individually, and used to construct prostate cancer specific metabolomic profiles based on the published PCA results (Cancer Res. 2005;65:3030-3034). The values of the calculated PCs for each voxel were used as indices in the color-map to determine the color and displayed transparently on an overlay of the anatomic image. The final metabolomic image is the anatomic map of the organ with the voxel grid overlaid—each voxel's color indicates that voxel's status on the cancer-index.

Results are summarized below. Metabolomic images, not images of a single or a few metabolites, present high intensities of cancer specific profiles in or around the regions where tumors are identified by histopathology. This observation is clinically significant for there is still no single test that can detect the locations of cancer even in a removed prostate before histopathology. However, when analyzing corresponding cancer indices between histology and metabolomic images for individual voxels, the correlation is non-linear. This can be caused by many reasons, such as intrinsic differences in experimental conditions between cancer profile determination and this pilot study; magnetic susceptibility effects at the surface of the specimens and out-of-voxel spectral contributions; and, the fixation of the specimens causes anatomic alteration that affects co-registrations.

Keywords: metabolomic profile, magnetic resonance, spectroscopy and Imaging

105 EPS Markers in the Early Detection of Prostate Cancer

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Expressed Prostatic Secretion (EPS) is obtained by milking the urethra following prostatic massage. Prostate cells released into EPS during prostatic massage are easily collected and analyzed. Our long-term goal is to exploit EPS as a noninvasive specimen in the diagnosis of prostate cancer. Our approach is to evaluate the relative performance of promising biomarkers in the prediction of prostate biopsy outcome.

Three types of biomarker have been compared: 1.) Hypermethylation of promoters represented by the APC, RARB, RASSFIA and GSTPI gene panel; 2.) Expression of prostate cancer specific gene fusions represented by the expression of Type III and Type VI TMPRSS2:ERG fusion RNAs; and 3.) Over-expression specific genes in prostate cancer cells represented by the over-expression $PCA3^{DD3}$ RNA. Quantitative PCR analyses developed for each biomarker employed the TaqMan® QPCR methodology referenced to linearized plasmid DNAs containing cloned representatives of the respective PCR target sequence.

Expressed prostatic secretion (EPS) was collected under an IRB-approved, blinded, prospective study from 74 patients undergoing trans-rectal ultrasound-guided biopsy for prostate cancer. The characteristic performance of each test in predicting biopsy outcome and distinguishing between high and low Gleason's Scores was compared. Logistic regression was used to analyze the effects of multiple biomarkers in linear combinations.

Each test improved characteristic performance over baseline DRE + Serum PSA, however, the test for Type III and Type VI TMPRSS2:ERG fusions yielded the best performance in predicting biopsy outcome (AUC=0.823 with a 95% CI [0.728-0.919]) and Gleason's grade>7 (AUC=0.844 with a 95% CI [0.740-0.948]). At 90% sensitivity, its NPV in predicting biopsy outcome was 0.810 with a 95% CI [0.612-0.898] and 0.820 with a 95% CI [0.553-0.907] for Gleason's Sum greater than 7.

Conclusions: 1.) While each test was shown to have diagnostic value, PSA + DRE + Type III and Type VI TMPRSS:ERG provided the best diagnostic performance in EPS specimens. 2.) EPS appears to be a valuable noninvasively-obtained specimen for prostate cancer diagnosis.

Keywords: expressed prostatic secretion (eps), biomarker performance, prediction of prostate biopsy outcome

106 A Fluorescence In situ Hybridization Screen for E26 Transformation-Specific Aberrations: Identification of DDX5-ETV4 Fusion Protein in Prostate Cancer

Bo Han, Rohit Mehra, Saravana M. Dhanasekaran, Jindan Yu, Anjana Menon, Robert J. Lonigro, Xiaosong Wang, Yusong Gong, Lei Wang, Sunita Shankar, Bharathi Laxman, Rajal B. Shah, Sooryanarayana Varambally, Nallasivam Palanisamy, Scott A. Tomlins, Chandan Kumar-Sinha, **Arul M. Chinnaiyan**

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Recurrent gene fusions involving E26 transformation-specific (ETS) transcription factors *ERG, ETV1, ETV4,* or *ETV5* have been identified in 40% to 70% of prostate cancers. Here, we used a comprehensive fluorescence *in situ* hybridization (FISH) split probe strategy interrogating all 27 ETS family members and their five known 5' fusion partners in a cohort of 100 clinically localized prostate cancer patients. Gene rearrangements were only identified in ETS genes that were previously implicated in prostate cancer gene fusions including *ERG, ETV1,* and *ETV4* (43%, 5%, and 5%, respectively), suggesting that a substantial fraction of prostate cancers (estimated at 30-60%) cannot be attributed to an ETS gene fusion. Among the known 5' gene fusion partners, *TMPRSS2* was rearranged in 47% of cases followed by *SLC45A3, HNRPA2B1,* and *C150RF21* in 2%, 1%, and 1% of cases respectively. Based on this comprehensive FISH screen, we have made four noteworthy observations. First, by screening the entire ETS transcription factor family for rearrangements, we found that a large fraction of prostate cancers (44%) cannot be ascribed to an ETS gene fusion an observation which will stimulate research into identifying recurrent non-ETS aberrations in prostate cancers. Second, we identified *SLC45A3* as a novel 5'fusion partner of *ERG*; previously, *TMPRSS2* was the only described 5' partner of *ERG*. Third, we identified two prostate-specific, androgen-induced genes, *FLJ35294* and *CANT1*, as 5' partners to *ETV1* and *ETV4*. Fourth, we identified a ubiquitously expressed, androgen-insensitive gene, *DDX5*, fused in frame with *ETV4*, leading to the expression of a DDX5-ETV4 fusion protein.

Keywords: gene fusion, prostate cancer, fusion protein

107 Tea Polyphenols in Chemoprevention of Prostate Cancer

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The anticarcinogenic potential of green (GT) and black tea (BT) has been demonstrated in many animal and *in vitro* cell culture studies. The major tea polyphenols of green tea are (-)-epigallocatechin gallate (EGCG) and (-)-epigallocatechin (EGC), whereas black tea contains smaller amounts of these polyphenols but contains larger polymeric flavonoids such as theaflavins and thearubigins. It has been demonstrated that GT and BT polyphenols inhibit cell growth through a variety of mechanisms such as antioxidant activity, alteration of redox-sensitive signal transduction pathways (nuclear factor kappa B, activator protein 1, mitogen-activated protein kinase) and inhibition of insulin-like growth factor (IGF-1) leading to the inhibition of proliferation, induction of apoptosis, cell cycle arrest as well as inhibition of angiogenesis. However most cell culture and animal studies have been performed with higher concentrations than achievable in humans. It is not clear whether effects observed in animal and cell culture studies can be applied to human studies. Therefore we are performing a phase II clinical intervention trial to investigate whether the consumption of 6 cups of GT or BT for 3-6 weeks prior to prostatectomy will decrease oxidative stress, alter signaling pathways leading to an inhibition of proliferation and increase of apoptosis in the prostate. Since in vivo polyphenols and theaflavins are subject to extensive endogenous and colonic metabolism we propose that metabolites contribute to the chemopreventive effect of GT and BT. Currently 23 participants have been enrolled. Using high performance liquid chromatography (HPLC) with coularray electrochemical detection as well as mass spectrometry (MS) EGC, EC and 4'-MeEGC were found in urine after GT consumption (0.8-2.3 μg/mL) and BT consumption (11-31 ng/mL). The majority was in glucuronidated form. After GT consumption EGC, EC, 4'-MeEGC, EGCG, 4'-MeEGCG and ECG were found in serum. EGC, EC and 4'-MeEGC were conjugated as glucuronide and small amount of sulfate, whereas EGCG, 4'-MeEGCG and ECG were mainly in the free form. Following GT and BT consumption urinary hippuric acid was increased. No theaflavins were found in urine or serum. EGCG was found in prostate tissue. No tea polyphenols were found in control participants. An interim analysis of immunohistochemical data on oxidative DNA damage, cellular proliferation, and apoptosis will be evaluated after 60 participants are enrolled. In vitro studies comparing the stability of methylated metabolite to parent compound demonstrated that methyl-EGC was stable at pH 7. Cell culture studies are being conducted to compare the effect on proliferation, apoptosis and NFκB DNA binding of metabolites to parent compounds. This phase II clinical trial will assist in the translation of *in vitro* and animal research to human application.

Keywords: green and black tea, prostate cancer, Phase II intervention trial

108 A First Generation Multiplex Biomarker Analysis of Urine for the Early Detection of Prostate Cancer

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Although prostate-specific antigen (PSA) serum level is currently the standard of care for prostate cancer screening in the United States, it lacks ideal specificity and additional biomarkers are needed to supplement or potentially replace serum PSA testing. Emerging evidence suggests that monitoring the noncoding RNA transcript *PCA3* in urine may be useful in detecting prostate cancer in patients with elevated PSA levels. Here, we show that a multiplex panel of urine transcripts outperforms *PCA3* transcript alone for the detection of prostate cancer. We measured the expression of seven putative prostate cancer biomarkers, including *PCA3*, in sedimented urine using quantitative PCR on a cohort of 234 patients presenting for biopsy or radical prostatectomy. By univariate analysis, we found that increased *GOLPH2, SPINK1,* and *PCA3* transcript expression and *TMPRSS2:ERG* fusion status were significant predictors of prostate cancer. Multi-variate regression analysis showed that a multiplexed model, including these biomarkers, outperformed serum PSA or *PCA3* alone in detecting prostate cancer. The area under the receiver-operating characteristic curve was 0.758 for the multiplexed model versus 0.662 for *PCA3* alone ($p = 0.003$). The sensitivity and specificity for the multiplexed model were 65.9% and 76.0%, respectively, and the positive and negative predictive values were 79.8% and 60.8%, respectively. Taken together, these results provide the framework for the development of highly optimized, multiplex urine biomarker tests for more accurate detection of prostate cancer.

Keywords: prostate cancer, urine, biomarker

109 Methylation Pattern of Multiple Genes in Urine Sediment DNA From Bladder Cancer Cases and Controls by Quantitative Methylation Specific PCR (QMSP)

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Background: The noninvasive identification of bladder tumors may improve disease control and prevent disease progression. Promoter methylation (i.e., hypermethylation) is now recognized as an important and common epigenetic pathway of gene inactivation. Because bladder cancer cells and DNA are shed into the urine, we sought to test the value of a quantitative methylation assay in urine sediment in patients suspected or harboring bladder cancer. **Methods:** A quantitative fluorogenic real-time polymerase chain reaction (PCR) assay was used to examine primary tumor DNA and urine sediment DNA from 15 patients with bladder cancer and 25 control subjects for promoter hypermethylation of nine genes (*APC, ARF, CDH1, GSTP1, MGMT, CDKN2A, RAR-β2, RASSF1A*, and *TIMP3*) to identify potential biomarkers for bladder cancer. We then used these markers to examine urine sediment DNA samples from an additional 160 patients with bladder cancers of various stages and grades and from an additional 69 age-matched control subjects. Data were analyzed on the basis of a prediction model and were internally validated using a jacknife procedure. All statistical tests were two-sided. **Result:** For all 15 patients with paired DNA samples, the promoter methylation pattern in urine matched that in the primary tumors. Four genes displayed 100% specificity. Of the 175 bladder cancer patients, 121 (69%, 95% confidence interval [CI] = 62% to 76%) displayed promoter methylation in at least one of these genes (*CDKN2A, ARF, MGMT*, and *GSTP1*), whereas all control subjects were negative for such methylation (100% specificity, 95% CI = 96% to 100%). A logistic prediction model using the methylation levels of all remaining five genes was developed and internally validated for subjects who were negative on the four-gene panel. This combined, two-stage predictor produced an internally validated ROC curve with an overall sensitivity of 82% (95% CI = 75 % to 87%) and specificity of 96% (95% CI = 90% to 99%). Using an optimal cutoff value, we found that the risk of death was statistically significantly higher in patients with higher *TIMP-3* and *ARF* methylation (HR 1.99, 95% CI 1.12 to 3.27, p = 0.01 and HR 1.66, 95% CI 1.00 to 2.76, p = 0.05, respectively) than in patients without/lower *TIMP3* and *ARF* methylation in urine. A significant correlation was also seen between the risk of death and stage 3 tumor (HR 2.73, 95% CI 1.58 to 4.72, p = 0.003) and metastasis (HR 3.32, 95% CI 1.98 to 5.57, p = 0.0001). Multivariate analysis subsequently revealed that TIMP-3 methylation was an independent prognostic factor for bladder cancer survival with stage and metastasis (p = 0.001 and 0.02, respectively).**Conclusion**: Testing a small panel of genes with the QMSP assay in urine sediment DNA represents a powerful noninvasive approach for the detection of bladder cancer on a high throughput-automated platform. *TIMP-3* promoter methylation could be a clinically applicable marker for bladder cancer progression. Larger cohorts with longitudinal follow up will be required in future studies to define the impact of this technology on early detection, prognosis and disease monitoring.

Keywords: bladder cancer, methylation, urine

110 A Tissue Biomarker Panel Predicting Systemic Progression after PSA Recurrence Post-Definitive Prostate Cancer Therapy

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Background: Many men develop a rising PSA after initial therapy for prostate cancer. While some of these men will develop a local or metastatic recurrence that warrants further therapy, others will have no evidence of disease progression. We hypothesized that an expression biomarker panel can predict which men with a rising PSA would benefit from further therapy.

Methodology/Principal Findings: A case-control design was used to test the association of gene expression with outcome. Systemic (SYS) progression cases were men post-prostatectomy who developed systemic progression within 5 years after PSA recurrence. PSA progression controls were matched men post-prostatectomy with PSA recurrence but no evidence of clinical progression within 5 years. Using expression arrays optimized for paraffin-embedded tissue RNA, 1021 cancer-related genes were evaluated – including 570 genes implicated in prostate cancer progression. Genes from 8 previously reported marker panels were included. A systemic progression model containing 17 genes was developed. This model generated an AUC of 0.88 (95% CI: 0.84-0.92). Similar AUCs were generated using 3 previously reported panels. In secondary analyses, the model predicted the endpoints of prostate cancer death (in SYS cases) and systemic progression beyond 5 years (in PSA controls) with hazard ratios 2.5 and 4.7, respectively (log-rank p-values of 0.0007 and 0.0005). Genes mapped to 8q24 were significantly enriched in the model.

Conclusions/Significance: Specific gene expression patterns are significantly associated with systemic progression after PSA recurrence. The measurement of gene expression pattern may be useful for determining which men may benefit from additional therapy after PSA recurrence. Such biomarker panels should be included in future clinical intervention trials in men with high-risk prostate cancer.

Keywords: biomarkers, prostate cancer, systemic progression

111 Soluble ErbB3 Levels in Bone Marrow and Plasma of Men With Prostate Cancer

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Prostate cancer has a propensity to metastasize to bone and tends to induce osteoblastic rather than osteolytic lesions. We have previously identified a soluble form of ErbB3, p45-sErbB3, in bone marrow supernatant from men with bone metastasis from prostate cancer. We subsequently showed that p45-sErbB3 has bone-forming activity (Lin et al. Oncogene 2008, in press). In this study, we sought to understand the clinical implications of the presence of soluble forms of ErbB3 (sErbB3) by establishing an enzyme-linked immunosorbent assay (ELISA) to detect sErbB3 levels in bone marrow and plasma samples from men with or without prostate cancer.

ELISA were performed on marrow samples from 108 men (34 with androgen-dependent [AD] disease, 30 with androgen-independent disease but negative bone scan [AI/BS-], and 44 with AI disease and positive bone scan [AI/BS+]); sequential marrow from 5 men during treatment; plasma from 52 men before and after one course of docetaxel treatment; and plasma from 95 men aged 70 or more with no evidence of prostate cancer.

A subset of men with clinically detectable bone metastasis has high sErbB3 levels. Within the AI/BS- group, men with higher sErbB3 levels seemed to have lower rates of bone metastasis. Similarly, in the AI/BS+ group, clinically detectable bone metastases took longer to appear in men with higher sErbB3 levels (median, 82 months) than in men with lower sErbB3 levels (median, 41 months). However, high levels of sErbB3 did not seem to confer a survival benefit after the development of metastasis. Among men with metastatic progression in bone, one docetaxel cycle treatment reduced plasma sErbB3 ($P < 0.0001$) but did not affect bone-specific AP ($P = 0.206$) or PSA ($P = 0.906$). sErbB3 is also detected in plasma from men with no evidence of prostate cancer.

The apparent correlation between higher sErbB3 levels and longer time to bone metastasis suggests that sErbB3 may participate in the progression of prostate cancer in bone.

Reference: Lin et al. Clin Cancer Res. 2008 14:3729-36.

Keywords: prostate cancer, bone metastasis, androgen-independent prostate cancer, bone marrow

112 Identifying Multiplex Expressed Gene Targets for Prostate Cancer Detection and Establishing a Prospective, Multi-center Case-Control Repository for their Validation at the Harvard-Michigan EDRN-CEVC

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Objective: We sought to identify expressed gene target candidates for early detection of prostate cancer, and to establish a case/control cohort repository that would provide a platform to validate these targets and subsequently serve as a NCI-wide resource for validation studies of biomarkers identified by others.
Methods/Results: Using the Affymetrix U133 array (Plus 2.0) to evaluate our radical prostatectomy tissue repository, we identified 1474 genes over-expressed in prostate cancer compared to normal prostate. Validation of these prostate cancer-associated genes by interrogation of publicly available prostate cancer array data (Lapointe et al, 2004) implicated 195 transcripts with concordant over-expression between the array datasets; further, 10 of these expressed genes were found to have signal peptide sequences indicating possible secreted protein products potentially detectable in urine, blood or other body fluids suitable for cancer screening. Next, to discern from among genes over-expressed in prostate cancer those genes that are NOT expressed at detectable levels in normal, adult human tissue apart from the prostate we conducted *in silico* interrogation of the largest publicly available dataset of gene expression in normal human tissues (Su AI et al, 2004). This combined *in vitro* and *in silico* strategy identified 75 prostate cancer-specific gene candidates for early detection or to target therapy. We then performed quantitative RT-PCR targeting each of the candidate antigens, and confirmed that 17 of the candidate genes were indeed over-expressed in prostate cancer (compared to normal prostate; $P < 0.05$ for each). Frequency of over-expression in prostate cancer for these antigens ranged from 57 % to 86%. Further, RT-PCR confirmed over-expression in prostate cancer among 6 of 10 genes ($p < 0.05$ for each) that included a signal peptide (indicative of putative protein secretion and detectability in body fluids). While conducting our transcriptome-wide analyses in vitro and in silico, we concurrently initiated a prospective, multi-center blood and urine repository that enrolled men with prostate cancer and cancer-free controls from 6 Urology practices in 3 states (Massachusetts, Michigan, and New York). All subjects underwent biopsy to ascertain whether prostate cancer was present. At time of analysis we have enrolled 1281 men in this repository, with serum, plasma, and peripheral blood cells collected in multiple aliquots for the entire cohort (n=1281) and cDNA isolated from post-DRE spun urine cellular fraction procured in 392 subjects. Cohort characteristics are tabulated:

Table 1. Characteristics of the Harvard-Michigan EDRN Prostate Cancer Case/Control Specimen Repository

Age - median (range)	Cancer Cases Controls	63 (42-93) 61 (38-83)
Race	Caucasian Other	88% 12%
Biopsy Result	Cancer No cancer	41% 59%
Cancer Stage	T1C ≥ T2	74% 26%
Cancer Grade	Gleason ≤ 6 Gleason ≥ 7	43% 57%

Conclusion: Our transcriptome-wide *in vitro* and *in silico* analyses have identified gene transcripts detectable in prostate cancer but not in non-prostate tissues. Our prospective case-control cohort provides a platform for validation of this multiplex signature and for other biomarker validation studies. **Funding:** NIH U01-CA11391

Keywords: prostate cancer, biomarker, RNA arrays

113 Androgen Dependence of Castration-Recurrent Prostate Cancer

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Introduction: An American man dies of prostate cancer (CaP) every 18 minutes. The expression of the androgen receptor (AR) and AR-regulated genes in CaP that recurs during androgen deprivation therapy (ADT) suggests a central role for AR in CaP growth despite low to undetectable levels of circulating testicular androgens.

Methods: The androgen-dependent CWR22 human CaP xenograft, TRAMP model of CaP, and fresh, frozen, microarrayed, and xenografted clinical samples of androgen-stimulated benign prostate, androgen-stimulated CaP, and castration-recurrent CaP were used to explore the role of the AR in recurrence of CaP during ADT.

Results: Neither AR gene mutations nor amplification of the AR gene can account for the majority of clinical cases of castration-recurrent CaP. 34 specimens of castration-recurrent CaP screened using denaturing gradient gel electrophoresis (DGGE) and direct sequencing identified an AR ligand binding domain mutation, a CAG repeat deletion and a glycine repeat deletion. AR gene amplification occurred in 8 of 24 patients, was associated with increased expression of AR, but did not impact survival. Instead, a number of mechanisms appear to contribute to AR transactivation in response to androgen. AR hypersensitization was first demonstrated using CaP "androgen-independent" cell lines where AR was transctivated by 10^{-15}M dihydrotestosterone (DHT). Mechanisms for AR hypersensitization to low levels of circulating androgen include increased levels of SRC/p160 coactivator expression and AR phosphorylation. The hypersensitive AR responds to intracrine synthesis of the bioactive androgens testosterone and DHT that may derive from circulating adrenal androgens. Tissue levels of testosterone were similar in castration-recurrent clinical samples of CaP and androgen-stimulated benign prostate. DHT levels, although lower, were sufficient to activate wild-type AR in most cases. To pursue the changes in androgen metabolism necessary to synthesize bioactive androgens from adrenal androgens, a mass spectrometry method was developed that allows simultaneous measurement of 7 androgens from small tissue specimens. One such change in androgen metabolism is that 5 alpha-reducing capability changes from the type 2 enzyme in androgen-stimulated benign prostate to type 1 in androgen-stimulated and castration-recurrent CaP. However, treatment of patients with castration-recurrent CaP with a 5 alpha-reductase bispecific inhibitor did not slow tumor progression. The origin of castration-recurrent CaP may be CaP stem cells that survive in the microenvironment perhaps facilitated by rapid reestablishment of the AR-regulated prostatic vasculature.

Conclusion: Innovative approaches to prevent intracrine synthesis of bioactive androgens, irreversibly degrade tissue androgens, degrade the AR, or target the prostatic vascular or stem cell niche may provide new therapeutic approaches to arrest the growth and progression of castration-recurrent CaP.

Keywords: prostate cancer, castration-recurrent, androgen receptor

114 Finasteride Attenuates the Association of Elevated C-Peptide With Increased Risk of High Grade Prostate Cancer: The Prostate Cancer Prevention Trial (PCPT)

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Metabolic abnormalities (including hyperinsulinemia) and obesity have been associated with increased cancer risk and/or worse cancer prognosis in several studies. C-peptide is a biomarker of pancreatic insulin production and higher levels are suggestive of hyperinsulinemia, which may lead to unfavorable downstream events, including up-regulation of the pAKT and mTOR pathways within neoplastic cells. Our recent laboratory research (eg. Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer xenografts. J Natl Cancer Inst. 2007 Dec 5; 99:1793-800) suggests a role for insulin in prostate cancer (PCa) biology. Having documented the presence of insulin receptors on PCa biopsy tissue, we recognized that there are opportunities for related translational research in both treatment and prevention contexts.

With respect to treatment, Phase I trials of insulin receptor–targeting agents are underway, and other modalities such as lifestyle modifications or metformin therapy are also being examined as strategies to reduce high insulin levels that may accelerate proliferation of androgen-independent PCa.

We report here early results in the prevention context, carried using the biorepository of the PCPT. The primary results of Phase III trials provide important evidence about the overall effectiveness of a drug on disease prevention or treatment with immediate clinical applications, but work with associated biorepositories can provide additional information concerning cancer biology and also can examine whether there are subgroups of individuals for whom the investigative drug may be particularly beneficial or harmful. The PCPT was a Phase III, randomized, double-blinded placebo controlled trial of the drug finasteride for the primary prevention of PCa. The goal of our research was to investigate whether C-peptide is associated with PCa risk, and in particular whether the risk differed for men taking finasteride vs. placebo. We used specimens from 1803 prostate cancer cases and 1797 controls. Case or control status for all participants was determined by sextant biopsy and central pathology review. C-peptide was assayed by ELISA. Higher vs. lower C-peptide concentrations were associated with a nearly two-fold increased risk of high-grade PCa (Gleason $\geq$ 7), but only among men in the PCPT placebo group. The multivariate high grade PCa cancer odds ratio for the fourth vs. first quartile of C-peptide was 1.90 (95% CI, 1.2-3.0). These estimates were adjusted for age, race/ethnicity, family history of PCa, use of insulin, body mass index and smoking. The data imply that hyperinsulinemia increases risk of high grade CaP, and that finasteride attenuates this risk. If confirmed, these findings have obvious clinical relevance.

Keywords: prostate cancer, finasteride, C-peptide, insulin

115 Aurora Kinase A as a Biomarker for the Detection of Bladder Cancer

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Background: Chromosomal missegregation resulting in aneuploidy is a common change in neoplasia, and genes regulating segregation of chromosomes may be potential cancer detection markers. Amplification/overexpression of aurora kinase A, a key regulator of mitosis, has been frequently detected in cancer and the degree of amplification correlates with the degree of aneuploidy.

Methods: We measured the level of aurora kinase A expression in bladder cancer cells and correlated it with the copy number of three selected chromosomes as well as total nuclear DNA content. The effect of aurora kinase A on centrosome multiplication and chromosome copy number was measured *in vitro* using an adenoviral expression construct. To assess the applicability of this gene as a biomarker for bladder cancer, we tested the aurora kinase A gene copy number using fluorescence *in situ* hybridization (FISH) on exfoliated cells from urine sediments of bladder cancer patients.

Results: Overexpression of aurora kinase A in urothelial cells induced amplification of centrosomes, chromosome missegregation, and aneuploidy. Moreover, overexpression of this kinase could be detected in *in situ* lesions as well as in urine of patients with bladder cancer. A FISH test for aurora kinase A gene copy number performed on a blinded validation test consisting of 100 voided urine samples from patients with bladder cancer and 148 controls detected bladder cancer with specificity 96.6% and sensitivity 87%.

Conclusion: Our findings indicate that overexpressed aurora kinase A can cause aneuploidy in urothelial cells and is a promising biomarker for detection of bladder cancer.

Keywords: Aurora A, biomarker, detection of bladder cancer

116 MDV3100, A Novel Androgen Receptor Antagonist for Castration Resistant Prostate Cancer (CRPC)

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Background: CRPC is characterized by persistent, high level androgen receptor (AR) expression and remains AR-dependent in model systems. MDV3100 is a novel small molecule AR antagonist that unlike bicalutamide, inhibits AR function by imparing nuclear translocation, DNA binding, has no agonist activity when AR is overexpressed, and induces regressions of established human LNCaP-AR xenografts in a dose dependent manner. In July 2007, a multi-center first-in-man Phase 1-2 trial was initiated to determine safety, pharmacokinetics (PK), and antitumor activity including changes in prostate-specific antigen (PSA), bone and soft tissue metastases, circulating tumor cell (CTC) number (CellSearch^{TM} (Veridex, LLC)) and in selected patients, fluorodeoxyglucose (FDG) and fluorodihydrotestosterone (FDHT) uptake by positron emission tomography (PET).

Methods: Pts were administered MDV3100 orally, once daily, starting at 30 mg with sequential escalations in cohorts of 3 pts. Enrollment was expanded at 60 mg and above once the safety of a dose was established. PK parameters for the dose-escalation cohorts were estimated using a 2-compartment model. Outcomes were reported using the Prostate Cancer Working Group Guidelines (JCO 26:1148, 2008).

Results: Accrual through the 240 mg/day dose level has been completed, and is ongoing at 360 mg/day. There have been no reports of serious adverse events attributable to study drug. PK including Cmax, Ctrough, and AUC24h are linear and the half-life is approximately one week. At 12 weeks, PSA declines of 90% or greater were observed in 9% (2/22), 13% (3/23) and 29% (8/28) of patients treated at 60, 150 and 240 mg per day, respectively, associated with stabilization of disease by imaging. ***CTC.*** For the 60 and 150 mg dose levels, favorable (Fav, 4 or less) and unfavorable (Unfav, 5 or more) counts were noted in 58% (25 of 43) and 42% (18 of 43) of cases. Following treatment, 92% of patients retained Fav counts, while 33% and 56% at 60 and 150 mg daily, converted from an Unfav to a Fav category. ***FDG and FDHT PET.*** Individual lesions are scored as 1, negative for tumor; 2, probably negative; 3, equivocal; 4, probably positive; and 5, certainly positive, and SUVmax recorded for lesions scored as 4 or 5. Four patterns were observed for the two tracers: concordant, glycolysis (FDG) predominant, AR (FDHT) predominant, and mixed, with at least one concordant lesion in all patients. To date, 8 pts have had FDG and FDHT PET at baseline of whom 4 have had 1 or more follow-up scans. All 8 had abnormal FDHT accumulation at baseline while 4 of 4 with follow-up scans had no FDHT accumulation. Decreases in FDG uptake in tumor were also observed. **Conclusions:** MDV3100 has been well-tolerated with encouraging anti-tumor activity assessed by: post-therapy PSA declines, imaging, the proportion of patients who are continuing on treatment, and biomarker changes including CTC, FDG and FDHT PET. The observed dose-response trends suggest these proportions may increase as higher doses are explored. Accrual is continuing. A structured Academic-Industry collaboration through the PCCTC can partner to develop promising therapies rapidly.

Supported by Medivation, Prostate Cancer Foundation, PCCTC and MSKCC SPORE in prostate Cancer.

Keywords: prostate cancer, androgen receptor, circulating tumor cells,, PET imaging

117 The Application of Imaging Mass Spectrometry in the Development of Biomarkers for Improved Clinical Decision Making in Prostate Cancer

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The overall goal of this EDRN-supported Biomarker Discovery Laboratory is the identification of tissue and fluids biomarkers that can be utilized in the detection and assessment of prostate cancer. The accomplishment of the guiding clinical goals established by our group requires a tightly integrated research team that spans expertise in basic, pathology and clinical sciences. This integration is fostered on the local level by the Virginia Prostate Center an entity populated by GU specialty clinicians, pathologists and basic researchers. The NCI EDRN provides for a similar integration strategy on the national scale. As an example of one possible model for the conduct of translational research we present our studies in the application of Imaging Mass Spectrometry to our efforts at discovery of prostate cancer biomarkers. The specific clinical objectives are to 1) Identify micrometastatic disease prior to surgical intervention, and, 2) Improve disease staging/grading by identifying insignificant/significant disease. We will present our core biomarker discovery efforts in the context of required supporting infrastructure. This model is of particular interest to groups that seek to balance highly technical, instrumentation-heavy, biomarker discovery approaches with biomarker advancement and near term clinical impact.

Keywords: prostate cancer, imaging mass spectrometry, biomarker

118 Signaling and Progression in Prostate Cancer

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Metastatic, hormone independent prostate cancer (CaP) is incurable. The goal of this multidisciplinary Program Project is to elucidate the signal transduction mechanisms that underlie the stepwise events associated with progression of CaP from a localized and androgen sensitive tumor to a disseminated and androgen independent one. The Program brings together productive and experienced investigators with complementary expertise relevant to the stated goal of the Program and backgrounds in signal transduction (J. T. Parsons, S. J. Parsons, Weber), nuclear receptor biology (Paschal), bone biology (Guise), human prostate cancer pathology (Frierson), biostatistics (Conaway) and basic and clinical prostate cancer metastasis research (Theodorescu). In Project 1, Theodorescu and J. T. Parsons propose to evaluate the roles of VEGF, FAK and Rap in CaP progression and metastasis to bone; Project 2, S. J. Parsons studies the regulation of neuroendocrine cell growth within advanced prostate cancers and the impact of such cells on overall tumor dependence on androgen; Project 3, M. Weber studies Ras-mediated signaling cascades as they affect ligand hypersensitive androgen receptor activity; Project 4, Paschal proposes to study the relationship between androgen receptor activation and the control of its nuclear localization. This interactive Program relies heavily on synergistic technical and scientific expertise from all investigators. The productivity of individual Projects is catalyzed by highly interactive Cores led by Theodorescu (Administrative Core A) which integrates the participation of M. Conaway an expert biostatistician; Guise (Cell, Animal and Imaging Core B) who has extensive experience in bone histology and histomorphometry and who is familiar with the biology of prostate cancer and the xenograft models used in prostate cancer research as well as their *in vivo* imaging; Frierson, (Tissue Analysis Core C), an expert surgical pathologist who specializes in CaP. Together, these Projects and Cores integrate diverse skills and expertise to focus on areas fundamental to our understanding tumor progression in CaP, with the objective of accelerating progress in developing a cure for this devastating disease.

Keywords: prostate neoplasms, metastasis, signal transduction

119 Tissue Effects of Selenium and Vitamin E in Prostate Cancer

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In two phase III trials, L-selenomethionine (selenium) and alpha-tocopherol (vitamin E) were shown in secondary analyses to reduce prostate cancer incidence. In a randomized, placebo-controlled phase IIA study correlative to the ongoing Selenium and Vitamin E Cancer Prevention Trial (SELECT), we sought to identify differentially expressed genes and to characterize the effects of selenium and vitamin E in normal epithelium, stroma, and tumor tissue.

Evaluated were 39 men with prostate cancer who were treated between diagnosis and prostatectomy (not >6 weeks) with SELECT levels of oral selenium (200 μg) and vitamin E (400 mg) daily. Laser-capture microdissection coupled with microarray was used to study ex vivo prostatectomy biopsy specimens and validated by real-time polymerase chain reaction. To identify genes that could discriminate between treatments and/or tumor types, we fit an ANOVA model. Pair-wise comparisons and contrasts were performed to assess differences between groups. The false-discovery rate (FDR) was estimated using a beta-uniform mixture model.

Normal epithelium, where 2109 genes were differentially expressed, was most affected by selenium (63%); stroma, where 2051 genes were differentially expressed, was most affected by vitamin E (66%); and tumor tissue, where 587 genes were differentially expressed, was most affected by the combination (56%) (FDR, 2%). Overall, we identified gene groups implicated in specific processes and major nodes of interaction in 11 networks and achieved a Spearson's correlation coefficient of 0.87 in tests of representative genes from each cell type differentially expressed.

Almost 600 differentially expressed genes were identified in all three tissue types and linked to specific molecular processes and characterizing networks. Results delineate the cell-type specific and zone-specific tissue effects of selenium and vitamin E. With these results, this model of tissue interrogation, the first of its kind, proves its efficiency, its feasibility, and its hypothesis-generating potential.

Keywords: prostate cancer, selenium, vitamin E

120 Biomarkers and Targeting the Reactive Stroma Microenvironment in Prostate Cancer Progression

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Our previous studies have shown that a reactive stroma initiates immediately adjacent to sites of PIN in early human prostate cancer genesis and co-evolves with cancer progression. Prostate cancer reactive stroma was characterized by accumulation of carcinoma-associated fibroblasts (CAFs) and myofibroblasts (MYFs) that express procollagen type I, fibroblast activation protein (FAP), and tenascin-C with an apparent displacement of normal smooth muscle. Considerable heterogeneity of reactive stroma grade was observed. Reactive stroma grade correlated significantly with progression. Patients with reactive stroma grade 3 progressed to biochemical recurrence after prostatectomy at a significantly higher rate than patients with reactive stroma grade 1 or 2. We have shown that TGF-β1 is overexpressed early in prostate cancer at some, but not all foci of PIN epithelial cells. To address mechanisms, the differential reactive stroma (DRS) xenograft model was constructed with LNCaP carcinoma cells combined with engineered prostate stromal cells. Human prostate stromal cells exhibited considerable heterogeneity in promotion of LNCaP tumorigenesis. Pro-tumor stromal cells exhibited elevated expression of CTGF, a downstream factor induced by TGF-β1 in human prostate stromal cells. DRS xenografts constructed with stromal cells engineered to overexpress CTGF exhibited elevated angiogenesis and tumorigenesis. DRS xenografts constructed with prostate stromal cells null for TGF-β receptor type II showed inhibited angiogenesis and tumorigenesis. Xenografts constructed with stromal cells engineered to overexpress Smad 3 dominant negative (ΔSmad 3) expression exhibited a similar attenuated phenotype. Prostate stromal cells null for TGF-β receptor II or overexpressing ΔSmad 3 also exhibited attenuated FGF-2 expression and release (secretion) of FGF-2. LNCaP xenografts constructed with prostate stromal cells that were both null in TGF receptor II (or positive for ΔSmad 3) and also engineered to overexpress FGF-2 exhibited rescued angiogenesis and tumorigenesis to near control levels.

In summary, these data suggest that TGF-β1 mediated signaling in reactive stroma through the TGF-β receptor II and in a Smad 3 mediated manner, functions to regulate multiple pro-angiogenic and pro-tumorigenic growth factors including FGF-2 and CTGF. Together, these factors influence the tumor microenvironment stromal cell phenotype and stimulate rates of angiogenesis, cancer cell proliferation, and overall tumorigenesis. As CAFs and MYFs appear as a generic component of most human carcinomas, and TGF-β1 is overexpressed in most epithelial cancers, targeted therapeutics to TGF-β signaling pathways in CAFs/MYFs may be a useful approach. Our recent studies have focused on addressing the putative marrow origin of CAFs/MYFs, as targeting the recruitment of progenitor cells may represent a therapeutic approach. As a component of the NCI Tumor Microenvironment Network (TMEN) our group has also evaluated reactive stroma markers in tissue arrays of mammary, colon, lung, prostate, pancreatic, thyroid and brain cancers. These studies are focused on assessment of common and different microenvironment cell phenotypes and expression of markers in the major human cancers, as common reactive stroma phenotypes and pathways could represent a general targeting opportunity for multiple cancers. Supported by R01 CA58093 and U54 CA126568.

Keywords: reactive stroma, transforming growth factor beta, tumor microenvironment

121 Large Scale-Evaluation of Candidate Genes Identifies Novel Low-Penetrance Bladder Cancer Susceptibility Loci

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Bladder cancer is a disease relevant to environmental exposure. However, there is compelling evidence that genetic predisposition plays an important role in its etiology. To identify and validate novel loci that contribute to bladder cancer, we applied a large scale evaluation of common genetic variations in 998 candidate cancer genes. Roughly 10,000 functional SNPs and tagging SNPs from these genes were genotyped in a Texas case control study with 805 pairs of newly diagnosed Caucasian bladder cancer patients and healthy controls. Thirty-one SNPs exhibited significant association with bladder cancer risk after adjusting for multiple testing with the q-value ($q<0.05$). The top two SNPs, rs1485762 in VEGF-C gene and rs3136329 in MSH6 gene, had p values <0.0001. Furthermore, a total of 10 SNPs were selected and genotyped for VEGF-C, of which 4 in addition to rs1485762 were associated with risk at the $p = 0.005$ and $q = 0.06$ levels. In haplotype analysis of these 5 SNPs, there was a trend of increased cancer risk with increasing number of variant allele. We also performed exploratory analyses of the cumulative effect and gene-gene interaction of the 31 significant SNPs in modulating bladder cancer risk. We found a significant gene-dosage relation between the number of putative adverse alleles and bladder cancer risk. Compared with the referent group ($\leq$11 adverse alleles), individuals with 12-15 (OR=4.74, 95% CI 2.66-8.47), 16-18 (OR=14.45, 95% CI 7.74-26.98), and $\geq$19 adverse alleles (OR=64.60, 95% CI 28.28-147.80) exhibited progressively increased risks of bladder cancer (P for trend $<10^{-7}$). In classification and regression tree (CART) analysis, we identified potential higher-order gene-gene interactions and categorized a few higher risk subgroups for bladder cancer. We also incorporated these novel loci into our previously developed bladder cancer risk prediction model and demonstrated that genetic variations can improve prediction efficiency of cancer risk. In conclusion, this largest candidate gene association study identified promising novel bladder cancer susceptibility locus and suggested a gene-dosage effect and gene-gene interactions in modulating bladder cancer risk. This study highlights that pathway-based candidate gene approach is still highly valuable and can complement genome-wide association study in identifying cancer susceptibility loci.

Keywords: bladder cancer, SNP, risk model

122 DNA Methylation Changes in Prostate Cancer: Promise as Biomarkers for Diagnosis and Risk Stratification

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Epigenetic gene silencing by CpG island hypermethylation occurs nearly universally in prostate cancer. These DNA methylation changes have tremendous potential as prostate cancer biomarkers for diagnosis and risk stratification. To further explore this potential, we have: i) identified novel biomarkers for prostate cancer diagnosis and prognosis using candidate gene approaches, ii) developed novel technologies for genome-wide discovery of DNA methylation biomarkers in prostate cancer, and iii) developed a novel technology for rapid, sensitive, and specific detection of DNA methylation biomarkers in prostate cancer relevant biospecimens. These findings represent the early stages of the TRWG pathway for biomarker development, including biomarker discovery and credentialing, and development of lead assays for biomarker detection. Next steps include early stage clinical trials to assess utility of these DNA methylation biomarkers in prostate cancer diagnosis and risk stratification.

Keywords: DNA methylation, biomarker, prostate cancer

123 TGF-Beta Induced Vimentin Expression Predicts Biochemical Recurrence Following Radical Prostatectomy

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Introduction and Objective: Over-production of TGF-beta by tumor-derived cells is considered to be involved in epithelial-mesenchymal transitions (EMT) in cancer cells. The role of EMT in prostate cancer remains unclear. We elucidate the relationship between TGF-beta signaling and EMT and its impact on the progression of human prostate cancer. **Methods**: Four human prostate cancer cell lines, including PC3-W (wild type), PC3-Pro (limited in prostate tissue), PC3-M (with metastasis) and PC3-LN4 (metastasis in lymph nodes) were treated with or without TGF-beta (1ng/ml/24 hours). As a negative control, these cells were rendered TGF-beta insensitive by transfection with a dominant negative TGF-beta type II receptor vector (TBRIIDN). Vimentin was used as a marker for EMT and was evaluated by immunohistochemical staining and Western blotting. Corresponding invasion assays were performed by the polycarbonate membrane method. We then studied 600 human prostate tissue specimens which included 100 benign specimens, 196 cancer specimens with low Gleason's sum (4-7), and 304 cancer specimens with high Gleason's sum (8-10). Tissue microarrays and immunohistochemical staining were performed on TGF-beta1 and vimentin. **Results**: Vimentin expression was significantly increased after treatment with TGF-beta, with more aggressive phenotypes (PC3-LN4 and PC3-M) demonstrating higher immunohistochemical vimentin expression. At least a 3.5-fold increase in protein expression was confirmed by quantified western blot when the cells were treated with TGF-beta. There is a dose-dependent relationship between the TGF-beta concentration and vimentin expression. Invasive cancer cells increased after treatment with TGF-beta, but decreased after transfection with TBRIIDN. Local TGF-beta expression was identified in 82.1%, 40.9%, and 19.1% of high and low grade cancers, and benign prostate tissue, respectively. Vimentin expression was identified in 86.6%, 48.1% and 9.5% of high and low grade cancers, and benign tissue, respectively. Furthermore, vimentin expression correlated directly with TGF-beta expression levels. Finally, both high expression of TGF-beta and vimentin correlated with more aggressive tumors (higher Gleason's scores). **Conclusions**: Over expression of TGF-beta by tumors will induce the expression of vimentin, which confers an aggressive phenotypic transformation. Assessing local TGF-beta signaling and expression of vimentin in cancerous tissue may be a novel method to evaluate prostate cancer prognosis.

Keywords: TGF-beta, Vimentin, recurrence

124 Diagnosis of Cancer With High-Resolution Ultrasound

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This effort focuses on the final development of a high-frequency (100 MHz), broadband ultrasound microsystem designed to image cellular structure/tissue along the gastrointestinal (GI) tract. The outcome of the current work is a production prototype. It consists of a paraboloid transmitter, a 16 × 16 ultrasound receiver array, a read-out integrated circuit (ROIC), and an interposer layer that links the latter two items. Fourteen-bit digitized data flow from the ROIC to an external computer where three-dimensional (3-D) images are formed. The nominal sampled volume extends below the GI tract surface to depths in the range 1-4 mm, depending on the proximity of the sensor to the GI wall. The primary images will be acquired in a conical volume that extends 1.0 to 2.2 mm in depth with corresponding cross sectional diameters of 0.6 mm and 0.9 mm, respectively. The radial resolution of the imager is ~9 μm. Lateral resolution is dependent on depth; it ranges from 26 μm at 1.0 mm depth (on axis) to ~60 μm at 2.2 mm depth (0.5 mm off-axis). The region of depth coverage can be split into several sample intervals to increase the range of depths. Within the observed volume, cells and tissue structure are imaged layer-by-layer, and an incisionless biopsy is effectively performed. The size of the sample volume is determined primarily by current ROIC technology; with increasing time this volume will increase. As it stands, this system is more than adequate for several key endoscopic functions. These include: 1) the imaging of pre-cancerous dysplastic mucosa, polyps, and adenomas, 2) real-time grading of dysplasia, 3) immediate viewing of cellular structure in tumors (e.g., squamous cell carcinoma and adenocarcinoma, benign growths), 4) guidance for directing fine-needle aspiration biopsies to regions that pose the greatest threat, 5) when used with lower frequency (5-12 MHz) endoscopic ultrasound, the imager prevents malignancies with large inflamatory components from being overstaged, 6) the unit aids in the detection and analysis of flat adenomas (often difficult to identify visually), and 7) the system serves as a patient friendly, pre-cancer diagnostic for severe gastroesophageal reflux disease (Barrett's esophagus), ulcerative colitis, and Crohn’s disease.

The developmental prototype has a novel bistatic architecture aimed at increasing system sensitivity, reducing the time required to obtain an image, and simplifying the receiver system. Two prototype arrays have been developed. A new sol-gel PZT thick film invented as part of our earlier research is used as one of two transducer materials. It is much improved over previous efforts in this area. The PZT thick film density is 89-90% of the full thin film density and the thick film piezoelectric coefficients are very close to those of thin films. In the past, our thick film single element transducers were successfully used to image biophysiological systems. The advantage of the PZT thick film is 1) its patterning and fabrication process is well established and many methods are available, 2) it supports an etch selectivity of 10:1, which is important for array definition, and 3) the fabrication/assembly process is straightforward and amenable to large-scale production. In addition, a more sensitive prototype system has also been constructed using single-crystal PMN-PT as the piezoelectric material. The fabrication process for both prototypes is similar and yields inexpensive transducer arrays. This is very important because the imaging sensor will be used only once and discarded. The difference between the PMN-PT and PZT units is that the PMN-PT has more than an order of magnitude increased sensitivity compared to PZT, and its etch selectivity is 20:1. However, the construction of the PZT imager is less complex than the PMN-PT system. The initial manufacturing cost for the PZT unit is estimated at $80, whereas the cost of the PMN-PT system is about $100. The ROIC is the dominant contributor to unit costs.

Keywords: high-frequency ultrasound, gastrointestinal tract, dysplasia

125 Hybrid Optoacoustic-Ultrasonic Modality for Diagnostic Imaging of Breast Cancer

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In 1880 Alexander G. Bell heard "a pure musical tone" in a closed gas volume that had absorbed a modulated sunlight beam. However, it would be a century before this discovery stimulated physicists to employ it in novel medical instruments. Recently, opto-acoustic tomography (OAT) emerged as a sensitive modality for visualization and quantitative characterization of malignant tumors and blood vessels. OAT combines the most compelling features of light and sound to provide maps of absorbed optical energy in optically scattering and opaque media including biological tissues. The new hybrid modality improves spatial resolution of the optical imaging and contrast of the ultrasound imaging.

The basic principles behind the optoacoustic imaging system are that (1) laser pulses may be effectively used to produce acoustic sources in tissues with enhanced optical absorption, and (2) ultrasonic waves propagate in biological tissues as expanding spheres with minimal wave-front distortion and deliver temporarily resolved information to the surface of tissue where it may be detected. The application of transducer arrays permits reconstruction of two-dimensional and three-dimensional images. One of the main endogenous chromophores of tissue in the near-infrared spectral range is the hemoglobin of blood. Therefore, blood vessels possess high optoacoustic contrast. Malignant solid tumors develop an enhanced network of microvessels to supply nutrition and oxygen to aggressively growing cancer cells. Therefore, optical contrast between normal and cancerous tissues is substantial allowing detection of small tumors at a depth of up to 40 mm in dense and heterogeneous breast tissue. Furthermore, functional information about hemoglobin concentration and its level of oxygen saturation in tumors can serve as a basis for noninvasive diagnostic utility of OAT.

The niche of OAT in medical imaging is to provide high-resolution 3D maps containing functional information on blood concentration, its oxygen saturation and water content in tumors. Combination of OAT with ultrasonic imaging, which provides morphological information on the tumor dimensions, shape and location, results in an advanced system for detection and noninvasive diagnostics of breast cancer.

Technical designs of the light delivery system, probes based on ultrawide-band ultrasonic transducer arrays and electronic data acquisition boards for two-dimensional and three-dimensional hybrid modality will be presented as well as the corresponding algorithms of signal processing and image reconstruction. The method and the results of the system calibration and validation in phantoms will be described. The system spatial resolution of 0.5 mm in the plane of 2D image and 1.5 mm resolution in 3D mode was demonstrated. Clinical studies performed in 29 breast cancer patients demonstrated that (i) OAT can detect tumors with high contrast in the dense breast using a safe level of laser illumination, (ii) the functional imaging capability of OAT provides radiologist with additional medically relevant information that enhances sensitivity and specificity of cancer detection.

Keywords: tumor angiogenesis, functional imaging, hybrid modality

126 Optical Technologies for Cervical Neoplasia

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We have formed a multidisciplinary group of clinicians, scientists, statisticians, epidemiologists, molecular biologists, behavioral scientists, and decision scientists to develop and assess optical technologies for the developed and developing world using the technology assessment paradigm of Littenberg: biologic plausibility, technical efficacy, clinical effectiveness, patient and provider outcomes, and cost-effectiveness, also called societal outcomes. Our group is experienced in performing clinical trials in the U.S., Canada, and Nigeria. We are leveraging funding from several sources to accomplish our goals. In the previous eight years of funding we have: held 14 conferences; written 150 manuscripts and 100 abstracts; and trained 100 undergraduate students, 50 masters' level students, 60 PhD students, and 20 post-doctoral fellows. We have evaluated quantitative cytology in Phase II trials in 1850 patients at three clinical sites, quantitative pathology of the cervix in Phase II trials of 1850 patients with 3765 biopsies at three clinical sites, fluorescence and reflectance spectroscopy using a point probe that measures 2 mm of tissue in Phase II trials in 1850 patients with 3765 biopsies at three clinical sites, and fluorescence and reflectance spectroscopy using a multi-spectral digital colposcopic approach that images the entire cervix in pilot trials (100 patients in five clinical sites). Behavioral research on the point probe and multispectral digital colposcope was conducted along with the clinical trials. Methodological work supported the trials. Similarly, data on accuracy and cost-effectiveness analyses of these four technologies are now ready and methodology reports have guided the direction of analysis.

Cervical cancer is the second most common cancer in women worldwide and the leading cause of cancer mortality for women in developing countries. When precancerous lesions are detected early they are easily treatable and cause no decrease in survival. Cervical cancer is a devastating disease and the treatment for more advanced stages is morbid, expensive, and ineffective. In the developed world we have good screening and detection programs, but these are expensive and require a well-developed infrastructure. In the developing world, where resources for screening are not available, many young women die of a preventable disease. Optical technologies provide a solution to these problems. Optical measurement of tissue provides quantitative information that can be analyzed, instantaneously producing an objective diagnosis even in the hands of a non-expert operator. Devices to make these measurements have become inexpensive, robust, and portable because of advances in related fields of engineering.

The innovative aspects of this program project are four-fold: 1) the project uses the cervix, a small and accessible organ for which the dysplasia-carcinoma sequence is well-understood as the basis for examining emerging optical technologies; 2) optical spectroscopy and quantitative cytologic and histopathologic analyses are evaluated for biological plausibility, effectiveness, acceptability, and cost-effectiveness in both the screening and diagnostic settings; 3) both technologies will have broad applications to other organ sites such as the oral cavity and lung, the digestive tract, the bladder, and skin; and 4) we have assembled a research network that is multidisciplinary, synergistic, and dedicated.

Keywords: cervical cancer, dysplasia-carcinoma, optical spectroscopy and quantitative cytologic and histopathologic analyses

127 Biomarkers Of Premalignancy And Detection of Early Lung Cancer

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Lung cancer is currently thought to result from a series of cellular and molecular changes in airway precursor cells that eventuate in invasive tumor. Characterization of these changes depends on the ability to access their site of origin in the lower airways and on application of cellular and molecular tests that may predict progression to invasive carcinoma. The Colorado EDRN Biomarker Development Laboratory has used a variety of tools to map the lower airway epithelium, characterize its histomorphology and test chromosomal and molecular lesions that may predict the future development of invasive lung carcinoma or prevalent invasive carcinoma. Assays for both blood and sputum have been tested, focusing mainly on central airway lesions.

In a series of 2,521 heavy smokers with obstructive airway disease who were observed over 9869 person years, we identified 174 incident carcinomas. Within this group, persons with sputum atypia of moderate grade or higher had an adjusted hazard ratio for developing lung cancer of 2.37. This figure may be considered a lower threshold in a search for biomarkers that could potentially predict future invasive lung carcinoma.

Individuals with moderate atypia have been offered white light and/or florescence bronchoscopy to better track changes in airway epithelium over time in an effort to identify molecular and cellular changes that may progress to lung cancer. To assist with this endeavor, the Colorado laboratory has worked with JPL/NASA to develop a web-based bronchial map that integrates imaging and other data from multiple platforms and provides a time/space framework for determining the fate of specific cell populations in high risk smokers. In preliminary studies we have been able to track lateral spread of squamous carcinoma *in situ* from specific foci in one lung through the airways to multiple foci in the contralateral lung over a period of two years. These data highlight the need to develop methods to treat “premalignant” lesions before they acquire the ability to spread and metastasize.

Molecular technologies that have been applied to airway epithelium have included tumor suppressor gene methylation and aneuploidy (FISH). Detection of sputum DNA methylation in 3 or more of 8 genes results in a hazard ratio of 4.5 at a time point 18 mos. before lung cancer diagnosis. Aneuploid detected in sputum cells using a multicolor probe set (LAVysion, Abbott Laboratories) at the same time point results in a hazard ration of 36. Additional methylation sites and FISH probes are currently being tested to improve the sensitivity and specificity of these assays. It appears that molecular assays can improve the predictive power of morphology alone but will probably benefit from further optimization of gene combinations and probe sets.

Finally, circulating biomarker assays are being developed to detect tumor cells in patients with existing carcinoma. The Colorado laboratory has concentrated for this effort on markers that are amplifiable and are strongly expressed in tumors but are absent in normal tissues and blood. Markers emerging from expression microarray analyses have included Cancer Testis genes (MAGE A, NY-ESO, TEX15 and XAGE1) and elongation factor EEF1A2 . This combination of markers distinguishes tumor homogenates from normal lung tissue with an AUC of 0.980. Whether this marker set will survive validation in sputum and peripheral blood and emerge as a significant early detection test is an open question at the moment. It seems likely that the marker set may have some value as a staging device but this use too will require additional validation.

Keywords: cytology, methylation, aneuploidy

128 Image-Based Markers of Breast Cancer Risk

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Breast density has been shown to be associated with the risk of developing breast cancer. The long-term goal of our research is to develop multi-modality, image-based markers for assessing breast density and parenchymal structure that may be used alone or together with clinical measures, as well as biomarkers, for use in determining risk of breast cancer. The general hypothesis is that inclusion of automated analyses of the parenchyma will improve the assessment of breast cancer risk.

We have performed image-based categorization of patient databases (mammographic and MR images) based on breast density, parenchyma morphology, and parenchyma kinetics. These various descriptors of breast density and parenchymal characteristics (i.e. image-markers) were then correlated with known indicators of risk (such as BRCA1 and BRCA2 deleterious mutations and presence of cancer on the contralateral breast).

Mammograms from 172 subjects: 30 women with the BRCA1/BRCA2 gene mutation and 142 low-risk women were retrospectively collected and digitized. As age is a very important risk factor, 60 low-risk women were randomly selected from the 142 low-risk subjects and age-matched to the 30 gene-mutation carriers. Our computerized mammographic analysis includes: (a) manual selection of regions-of-interest from the central breast region behind the nipple and (b) mammographic parenchymal texture analysis to yield measures related to the spatial frequency content of the parenchymal morphology. Texture analyses performed included Fourier and power spectrum analyses and fractal analyses. The performance of the various texture features were used as decision variables for differentiating between gene-mutation carriers and low-risk women using receiver operating characteristic (ROC) analysis, with AUC values of 0.90 being achieved.

From the mammographic analysis, we have found that women at high risk for breast cancer have dense breast and parenchymal patterns that are coarse and low in contrast. We have also validated these results, which were initially found from our digitized screen/film mammography studies, on cases from full-field digital mammography.

We also investigated breast parenchymal enhancement on DCE-MRI and related the analyses to breast density. Our computerized breast parenchymal analysis includes: (a) automatic segmentation of the 3D breast volume from DCE-MRI images using a volume-growing based algorithm; (b) classification of the extracted breast volume into fibroglandular region and fatty region using fuzzy c-means/thresholding; (c) extraction of the parenchymal kinetic curves of the breast fibroglandular region and categorization into using fuzzy c-means clustering; (d) extraction of various kinetic features from the most enhancing voxels. MR images used in this study were acquired using a standard double breast coil on a 1.5 T GE whole-body MRI system with the patient in the prone position. One pre and five post-contrast DCE-MR images were obtained using a T1-weighted 3D spoiled gradient echo sequence with no fat suppression. Our preliminary study contained 54 DCE-MRI exams of asymptomatic women with normal MR findings. 85% (46/54) of the highest enhancing curves reached peak intensity at either 4^{th} or 5^{th} post-contrast time points. Women with dense breast (BIRADS 3 and 4) were found to have more parenchymal enhancement at peak time point with an average Ep of 101.4% than those women with fatty breast (BIRADS 1 and 2) with an average Ep of 75.0%. We found that parenchymal enhancement may be associated with breast density and may be potentially useful for assessment breast cancer risk.

In the future, it is expected that such image-based markers will be useful for improved assessment of patients at high risk for breast cancer and for monitoring the response of preventive treatments.

Keywords: breast cancer risk, mammography, MRI, texture analysis

129 Evaluation Of Lung Cancer Chemopreventive Agents In Phase II Clinical Trials

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Serial bronchial biopsies are currently used to sample preneoplastic lesions to evaluate the effect of chemopreventive agents in Phase II clinical trials. The biopsy procedure itself can potentially introduce artifacts by mechanically removing these lesions. It is therefore important to develop non-biopsy methods that can determine the presence and progression/regression of preneoplastic lesions in the bronchial epithelium. Optical coherence tomography (OCT) is an optical imaging method that can offer microscopic resolution for visualizing cellular and extra-cellular structures at and below the bronchial surface. Bronchoalveolar lavage (BAL), performed in the same bronchoscopic procedure also allows measurement of biomarkers associated with regression or progression of preneoplastic lesions. OCT images and the corresponding bronchial biopsies were obtained from current and former smokers during autofluorescence bronchoscopy as part of Phase II trials. The results showed that invasive cancer can be distinguished from CIS and that dysplasia can be distinguished from metaplasia, hyperplasia or normal. Using quantitative measurement, a progressive increase in the epithelial thickness was found to parallel the severity of the histopathology grade. The nuclei of the cells became more discernible in lesions that are moderate dysplasia or worse. CC10 and surfactant protein D levels in the BAL fluid were found to be significantly correlated with regression/progression of bronchial dysplasia. Our study suggests that OCT is a promising non-biopsy tool for in-vivo imaging of pre-neoplastic bronchial lesions to study their natural history and the effect of chemopreventive intervention. Measurement of constitutents in BAL fluid can be informative regarding the effects of chemopreventive agents.

Keywords: optical imaging, biomarkers, chemoprevention

130 Molecular Fluorescent Imaging for the Early Detection of Colorectal Neoplasia

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When colorectal cancer (CRC) is detected at an early stage, the 5-year survival exceeds 95%. Although colonoscopy is considered the current gold standard for screening, there is a miss rate for polyps as high as 22% using back-to-back colonoscopies. Additionally, "flat lesions" are more readily missed and may be more likely to contain dysplasia. The problem is further exacerbated in ulcerative colitis (UC), in which dysplasia can develop in macroscopically normal-appearing mucosa. Current colonoscopic surveillance in UC patients relies on random biopsies throughout the colon, which is a relatively insensitive and cumbersome strategy that is not widely utilized. Several techniques (e.g. chromoendoscopy) are actively being investigated to reduce lesion "miss rates" and improve detection of "flat adenomas" and UC-associated dysplasia. However, non-specific targeting of neoplastic tissue, poor correlation with histological findings, and prolonged exam time limit these approaches. Therefore, novel technologies that allow early, unequivocal detection and in situ characterization of colonic lesions with high sensitivity and specificity are needed.

We have developed a set of new technologies that utilize novel near infrared (NIR) activatable optical probes selective for proteases often overexpressed in tumors combined with quantitative NIR endoscopic imaging devices to enhance the detection of colonic neoplasia. We have shown that cathepsins are upregulated in the entire spectrum from dysplasia to invasive carcinoma and can serve as molecular targets for these optical probes. We have designed and optimized such probes, as well as novel macroscopic and microscopic technologies to image these agents in vivo. Specifically, in murine models, we have shown that colonic NIR imaging after probe injection results in a target-to-background (TBR) of approximately 9:1 in adenocarcinomas with a sensitivity and specificity for adenomas of 96% / 93%; in contrast, white light imaging results in a TBR of 1:1 for adenocarcinomas with a sensitivity and specificity of 49% / 40% for adenomas. Similarly, in a chemically induced murine UC model, colonic NIR imaging maintained a TBR greater than 8:1 for adenocarcinoma, despite the presence of adjacent inflammation. We have recently shown that these findings hold in more realistic preclinical mouse models, including the novel lox APCΔ580, which may more closely mimic human disease through the focal induction of colonic lesions. Our future plans focus on leveraging our extensive preclinical work into a pilot clinical trial in which the feasibility and diagnostic performance of this novel technology will be evaluated in patients with sporadic invasive CRC, patients with polyposis syndromes, and patients with dysplasia in the setting of UC.

Keywords: colorectal cancer, screening, molecular imaging

131 Breast Cancer Diagnostics Based On Interphase Spatial Genome Positioning

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The genome is non-randomly organized within the three-dimensional space of the cell nucleus. The nuclear position of many genes and genomic regions changes during physiological processes such as proliferation, differentiation and, importantly, disease. We hypothesize that we can exploit the changes in gene positioning patterns as indicators of disease to develop a novel diagnostic tool in the detection of breast cancer. To this end, we have analyzed the spatial position of a defined set of cancer-associated genes in an established mammary epithelial 3D cell culture model of early stages of breast cancer. We find that the genome is globally reorganized during normal and tumorigenic epithelial differentiation. Systematic mapping of changes in spatial positioning of cancer-associated genes reveals gene specific positioning behavior and we identify several genes which are specifically repositioned during tumorigenesis. We have extended these studies by comparing the positioning patterns of 20 genes in normal and tumor breast tissues. We have identified a set of marker genes which exhibit differential positioning in malignant tissues. Moreover, we have identified positioning markers for distinguishing different tumor types. Our results represent proof-of-principle for the application of spatial genome positioning patterns as a novel strategy for cancer diagnosis.

Kaywords: breast cancer, chromosome, nuclear architecture

132 Cone Beam Breast CT Imaging

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Koning Corporation

The long-term goal of this project is to develop and commercialize a novel cone beam breast computed tomography system to provide clinically useful 3D high-resolution tomographic images for breast cancer detection, diagnosis and treatment. According to the National Cancer Institute, 1 out of 8 women will be diagnosed with breast cancer in their life. While a reduction in mortality from breast cancer is evident in published reports, >200,000 new breast cancer cases occur and 40,000 women will die of the disease each year in the US. Mammography, which is the current standard care for breast cancer screening, misses 30% of breast cancers. Specificity and the positive predictive value of mammography remain limited owing to an overlap of benign and malignant tissue. Limited sensitivity and specificity in breast cancer detection of mammography are due to its poor contrast detectability, which is common for all types of projection imaging techniques (projection imaging can only have up to 10% contrast detectability). The average size tumor that can be detected by mammography is 11 mm. At best, it appears that mammography can reduce the death rate by up to 50%. While this is an important gain, considerable room still exists for improvement.

To address the limitations accompanying mammography, Koning Corporation is developing a Cone Beam Breast CT system, called Koning Breast CT (KBCT) which combines the advantages of digital x-ray and CT. KBCT technology utilizes a cone-shaped x-ray beam and a digital flat panel detector for volumetric data capture of a breast to produce clinically significant 3D images of breast anatomy with high and isotropic resolution. A research prototype of KBCT for diagnostic breast imaging was designed by Koning Corporation and constructed at the University of Rochester. A series of phantom and small animal studies have been performed on the KBCT system to evaluate and optimize the technique and the system. .The results of the study indicate that KBCT can detect ~2-3 mm tumor and ~0.2 mm calcifications with glandular dose equivalent to that of diagnostic mammography. A pilot study clinical trial at the University of Rochester has been performed for both normal and malignant cases without contrast agent. Initial clinical results indicate that the coverage, image quality and average glandular dose to the breast is comparable to conventional diagnostic mammography, while occult masses are more readily seen due to its tomographic imaging that removes overlapping structures. Based on the results of phantom, small animal and initial clinical trials, the design of KBCT system was optimized and a first commercial grade KBCT, Koning CBCT 1000 for diagnostic breast imaging was constructed which passed Electromagnetic Compliance and Electrical Safety Testing by a Nationally Recognized Testing Lab. This is an essential milestone in obtaining FDA approval for the medical device. The first Koning CBCT 1000 was installed at the Elizabeth Wende Breast Care, LLC and a pilot study has been conducted for both normal and malignant cases without contrast agent and with contrast agent (for cancer cases only). The results of the pilot studies will be presented. Potential technical advantages of KBCT over mammography: 1. Detect smaller carcinomas by eliminating lesion overlap and superimposed structures with up to 20 times better contrast detectability; 2. Better tumor characterization with true 3D visualization and volume of interest reconstruction for accurate measurement of size, volume and density of breast tumors; 3. Distinguish benign from malignant tumors using a volume growth measurement technique, with IV-injected contrast enhancement.

Koning has created a new modality of diagnostic breast imaging, Koning CBCT 1000. A logical extension of our current KBCT project for diagnostic breast imaging is to move from Creation of Modality Stage into Preclinical Development Stage and to perform clinical trials to validate the technology, achieve FDA approval for marketing the diagnostic breast imaging system in US and promote wide clinical use. Unlike mammography, mainly used for breast cancer detection, KBCT imaging is a platform breast imaging technology that can be used for breast cancer detection, diagnosis and treatment. It is expected that KBCT will significantly improve sensitivity in the detection of otherwise occult tumor and disease extent, while improving specificity (potentially reducing overall biopsy rates). It will also be a platform for minimally-invasive procedures, such as KBCT-guided biopsy, and treatment procedures, such as lesion ablation. Screening and minimally-invasive procedures will be funded by subsequent projects.

Keywords: breast-cancer diagnosis, breast-cancer treatment, cone-beam CT

133 Imaging the Breast With Alternatives to Conventional Practice

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We are investigating four experimental imaging methods (1) Magnetic Resonance Elastography (MRE), (2) Electrical Impedance Spectroscopy (EIS), (3) Microwave Imaging Spectroscopy (MIS) and Near Infrared Spectral Imaging (NIR) to determine if they can contribute to breast imaging for (a) risk assessment, (b) early detection, (c) differential diagnosis, (d) treatment prognosis and (e) therapy monitoring. We are also developing co-registered, multi-modality platforms that synergistically fuse the image data from these alternatives with standard methods such as breast MR. The work is motivated by the fact that the detection of breast cancer at an early stage increases the likelihood of successful treatment and long term survival, and yet, is underscored by the recognition that not all cancers will be detected early and imaging methods are needed to inform clinical intervention as well.

Clinical study designs which target: (1) the imaging of screening abnormalities recommended for biopsy, (2) the imaging of palpable masses on clinical breast exams, and (3) the imaging of locally-advanced cancers receiving neoadjuvant therapy are underway. Recent results report an evaluation of 150 participants from a population of women with screen-detected abnormalities. Image property contrast ratios of 150%–200% were found in breast abnormality regions of interest (ROIs) relative to the ipsilateral breast background. Receiver operating curve (ROC) analysis of the alternative image properties for cancers among subjects with BI-RADS category 4 or 5, compared with the same image properties for the subjects with normal breasts (BI-RADS category 1), yielded areas under the ROC curve ranging from 0.67 to 0.81. Pathologic correlations with mean vessel density, mean vessel area, and epithelium-to-stroma ratio suggest a biological origin of the alternative image properties associated with disease.

Quantitative changes occurring in breast tissue during neoadjuvant chemotherapy have also been monitored. ROI data in the tumor and surrounding breast were evaluated 5 or 6 times during chemotherapy and compared to contrast enhanced MR imaging and mammography at pre- and post- therapy time points. Despite significant structural and compositional changes occurring in the breast during the course of therapy, pilot data indicates that it is possible to track tumor response through quantification of alternative image property changes, and importantly, the magnitude of the property changes appeared earlier in the course of therapy than did corresponding ROI size changes.

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Keywords: breast imaging, nonlinear image reconstruction, functional signatures

134 Translation of Radiolabeled Prostate Stem Cell Antigen (PSCA) Antibody Fragments to the Clinic

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Purpose of study: Prostate Stem Cell Antigen (PSCA) is a cell surface glycoprotein expressed in normal prostate and overexpressed in prostate cancer including androgen-independent disease. Engineered antibody fragments including diabody (scFv dimer, 50 kDa), minibody (scFv-C_H3 dimer, 80 kDa) and scFv-Fc (105 kDa), were evaluated for their ability to target image androgen-dependent and -independent prostate cancer xenografts. ^{124}I-labeled hu2B3 minibody provided optimal imaging at 21hr and was selected for GMP production and translation into first-in-human trials, which are planned for Spring, 2009.

Experimental procedures: Anti-PSCA diabody, minibody and scFv-Fc were engineered from hu1G8, characterized biochemically, and assessed for PSCA binding by ELISA. Tumor targeting and imaging were evaluated in SCID mice bearing LAPC-9 xenografts. Serial microPET imaging was performed using ^{124}I-labeled fragments, and tumor uptake and target:background ratios were determined. In addition, affinity-matured versions of the 2B3 minibody were generated by yeast display. Following error-prone PCR, three clones were identified with improved binding to PSCA. These affinity-matured variants were reformatted into minibodies and tested against the parental minibody for tumor uptake and imaging performance. An affinity matured minibody was selected for clinical translation based on comparative imaging studies. High expressing clones (40mg/L) were developed for GMP production and cell banks created.

Results: MicroPET imaging revealed that the 80 kDa 2B3 parental minibody fragment performed best in LAPC-9 xenografts at 21 h. The parental minibody showed superior tumor uptake (5.2 %ID/g) compared to the diabody (0.7 %ID/g), and a higher tumor-to-blood ratio compared to the scFv-Fc (1.1 vs 0.6). Furthermore, PC-3-PSCA and LAPC-9 androgen-independent xenografts were also successfully imaged using the 2B3 minibody. Of the affinity matured variants, A11 achieved the highest tumor uptake (6.4 %ID/g) and tumor-to-blood ratio (1.1) at 22 h. Compared to the parental minibody, A11 showed a 26% higher tumor-to-blood ratio and a 141% increase in the tumor:background in LAPC-9 SCID mice.

Conclusion: The anti-PSCA minibody emerged as the optimal engineered antibody format, decreasing the imaging time to 21 h from 168 h post injection. These studies demonstrate the utility of an affinity matured, PSCA-specific minibody for imaging androgen-dependent as well as androgen-independent prostate cancer. The A11 minibody has been selected for clinical translation. High expressing clones have been developed and cell banks created for GMP production at the City of Hope. A first-in-human study is planned for Spring, 2009.

Keywords: minibody, prostate cancer, molecular imaging

135 Rectal Optical Signatures as a Marker for Colonic Field Carcinogenesis: Applications for Minimally Intrusive Mechanism for Risk Stratification

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Background and Aims: Colonoscopy represents an excellent means of preventing colorectal cancer (CRC) by identification and removal of adenomatous polyps. However, given resource constraints and patient reluctance, only ~20% undergo colonoscopy. Moreover, only ~5% of colonoscopies yield significant lesions. Given that the vast majority of colonoscopies are unnecessary, risk stratification for colonoscopy is critical. Field carcinogenesis detection represents common means of assaying distal (most easily accessible mucosa) to predict risk in the entire colon although most conventional markers (e.g. adenoma on flexible sigmoidoscopy or rectal aberrant crypt foci) lack adequate sensitivity. The novel optical technology, low-coherence enhanced backscattering spectroscopy (LEBS), allows identification of nanoscale architectural consequences of the field carcinogenesis in pre-clinical CRC models with unprecedented accuracy. We now assess potential clinical applicability.

Methods: LEBS markers were calculated from biopsies obtained from the normal-appearing rectal mucosa from patients undergoing colonoscopy (n=219) via a bench-top instrument. A pilot study (n=30) was then performed with a novel endoscopically-compatible optical probe to measure rectal LEBS markers *in vivo*.

Results: We observed that, in general, LEBS signal parameters mirrored neoplasia progression from patients with no dysplasia, to 5-9 mm adenoma and to advanced adenomas. An LEBS marker calculated from the LEBS signal paralleled this risk status (ANOVA $p<0.001$). Moreover, this was independent of CRC risk factors, benign colonic findings or clinically-unimportant lesions (diminutive adenomas, hyperplastic polyps). For advanced adenomas, the LEBS marker had a sensitivity =100%, specificity =80% and area under the receiver operator characteristic curve=0.895. Cross-validation with leave-one-out analysis showed a comparable AUROC. Moreover, a small independent validation set of convenience showed AUROC that was almost identical to the main dataset (0.70 vs 0.71 for all adenomas which included a diminutive). There was no evidence of confounding from demographic factors (gender, age, smoking/alcohol status etc) or benign colonic findings (hyperplastic polyps, diverticulosis etc). LEBS *in situ* analysis with novel endoscopically compatible optical probe was able to accurately determine spectral markers. The discrimination for non-diminutive adenomas versus control appeared to be excellent with a sensitivity, specificity and AUROC of 100%, 94% and 0.956 respectively.

Conclusions: We provide the first demonstration that LEBS-detectable alterations in the endoscopically normal rectum were associated with the presence of neoplasia located elsewhere in the colon. This study provides the proof of concept that rectal LEBS analysis provides an accurate gauge of concurrent neoplasia. Importantly, the negative predictive value was excellent indicating that this approach may eliminate many unproductive colonoscopies. Ongoing studies include large-scale multi-center validation protocols and assessment of the ability of this novel approach to gauge familial CRC risk.

Keywords: colon cancer, colorectal cancer, carcinogenesis

136 Tactile Imaging of the Breast: Cancer Screening and Diagnostic Potential

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Tactile Imaging (TI) is an emerging medical diagnostic technology for visualization of internal structure of tissues in terms of their elastic properties [1]. With support of NIH grants, Artann Laboratories has developed TI devices for prostate imaging and for breast cancer screening, as disclosed in 11 issued and 7 pending patents. The clinical work performed is Phase II of the NIH supported SBIR grant on Tactile Breast Imager (TBI), was an important step towards a long-term goal: development of a cost-effective, sensitive, easy-to-use and portable device for detection and differentiation of cancerous and benign lesions in the breast. This device may quantitatively evaluate multiple mechanical and structural properties of breast and breast lesions, such as Young's modulus, elasticity contrast, nonlinearity (strain hardening), heterogeneity index, nodule size, shape and mobility, which could be altered by cancer development.

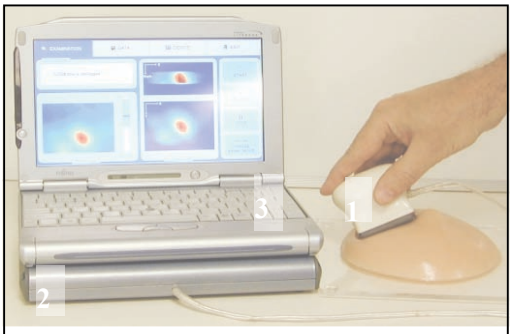

Figure. A.1. *General view of Tactile Breast Imager. The device comprises (1) a probe with 2-D pressure sensor array, (2) an electronic unit, (3) a laptop computer with touch screen capability.*

Clinical results collected at four different clinical sites for 187 cases have demonstrated TBI capability for real time characterization and differentiation of benign and malignant breast lesions [2-4]. Characteristic elasticity patterns and combinations of lesion features for specific breast pathologies were identified. Histologically confirmed malignant breast lesions demonstrated increased hardness and strain hardening as well as decreased mobility and changes in the relative boundary length in comparison with benign lesions. Statistical analysis of the TBI differentiation capability for 154 benign and 33 malignant lesions revealed an average sensitivity of 89.4% and specificity of 88.9% with a standard deviation of ±7.8%. The study had indicated TBI potential for a cost effective device for cancer diagnostics that could be positioned as an adjunct to mammography and utilized as a screening device for breast cancer detection. The next step in TBI development is performance of the Phase II clinical studies for in-depth technology validation and FDA application.

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Keywords: imaging, breast cancer, cancer screening and diagnostics

137 The Pittsburgh Lung Screening Study (PLuSS): A Research Cohort of Current and Former Cigarette Smokers Screened for Lung Cancer with Helical Computerized Tomography (CT)

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Estimated annual age-adjusted (U.S. 2000 standard) lung cancer mortality rates in current and former cigarette smokers are 2.2 and 5.9 per 1000 in men and 1.4 and 4.0 per 1000 in women. Between 2002 and 2005, no more than one out of five new primary lung cancer cases in 50-79 year-old persons were localized stage at diagnosis (17-registry SEER). Five-year relative survival for 50-79 year-old persons with lung cancer diagnosed between 1998 and 2000 (13-registry SEER) was 16% for all stages, but 52%, 21%, and 3% for localized, regional, and distant stages, respectively. Moreover, screening asymptomatic persons with computed tomography (CT) can detect early stage lung cancer with favorable long term prognosis (I-ELCAP. New Engl J Med 2006;355:1763). These observations motivate efforts to develop CT for early lung cancer detection.

Between March 2002 and September 2005, the Pittsburgh Lung Screening Study (PLuSS) used low-radiation-dose CT to screen N=3642 50-79 year-old current and former cigarette smokers (median 47 pack-year smoking history). Baseline activities included a risk factor questionnaire, peripheral blood sample collection, pulmonary function testing (PFT), and physician referral for CT-detected non-calcified lung nodules. One year after the initial screen, N=3423 subjects returned for a repeat CT screen.

PLuSS referred N=1726 (47% of 3642) subjects because of non-calcified lung nodule(s) detected on either initial or repeat CT. In the entire cohort, follow-up (extending two years after the repeat screen or, for subjects without a repeat screen, three years after the initial screen) identified 80 subjects (2.2% of 3642) with lung cancer (11 small cell and 69 non-small cell, including 40 stage I). N=36 subjects (1.0% of 3642) referred for CT-screen abnormalities had a major thoracic surgical procedure (thoracotomy, video-assisted thoracoscopic surgery - VATS, median sternotomy, or mediastinoscopy) that produced a non-cancer final diagnosis. Out of 82 subjects with thoracotomy or VATS to exclude malignancy in a lung nodule, 28 (34.1%) received a non-cancer final diagnosis.

The frequent CT detection of lung nodule and the risk of unnecessary surgery triggered by CT screening motivate efforts to identify effective methods for stratifying current and former smokers according to lung cancer risk. In PLuSS, a logistic regression model that included sex, years of age, years of cigarette smoking, smoking intensity (cigarettes/day in four categories), severity of airflow obstruction on PFT (in four categories), and severity of visually scored emphysema on CT (in four categories) predicted lung cancer (area under the curve = 0.75) with 20-fold risk stratification (10% *vs*. 0.5% expected lung cancer risk in the highest risk decile *vs*. lowest risk decile). Moreover, CT detection of radiographic emphysema predicted lung cancer (odds ratio 3.14, 95% confidence interval 1.91-5.15), independently of sex, age, smoking history, and PFT results.

The promise of lung cancer predictive models with recognition of radiographic emphysema as an independent risk factor motivates current efforts: 1) to continue long term follow-up of the PLuSS cohort, 2) to develop emphysema measures based on computer analysis of CT image data, and 3) to collect, at yearly intervals in a high risk subset of the original PLuSS population, additional biological samples (blood and sputum) and PFT data. In the setting of CT screening, the enhanced PLuSS resource enables translational research directed toward the development of more accurate lung cancer predictive models that use precise measures of emphysema severity, genetic measures of susceptibility, biomarkers of early disease, and temporal change in lung function.

Keywords: lung cancer, computed tomography, screening

138 Multi-Wavelength Confocal Imaging for Skin Lesions

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The Institute of Optics

Confocal laser scanning microscopy (CLSM) images the tissue and cellular morphology in-vivo by collecting and displaying the light reflected and scattered from a thin section within tissue. Our goal is to determine the capability of a multi-wavelength CLSM to image the key morphologic features of a range of pigmented and non-pigmented lesions such as nuclear atypia and formation of cancer nests including cancer-to-normal dermis margins, and distinguishing those from other potentially confounding normal features such as hair follicles, cysts, inflammatory cell nests and fibroblasts. Single-wavelength CLSM has demonstrated the ability to image the superficial layers of the epidermis, and the ability to detect tumor cells, nests and margins with a resolution of <5 micrometers. Multi-wavelength CLSM is technically equivalent to previous generation reflectance confocal microscopes. However they offer the ability to capture reflectance images sequentially at one of three wavelengths (785, 810, 850 nm) in rapid succession (0.1 sec between images). The wavelength-dependent scattering of various cellular and tissue components can be compared by image normalization and subtraction. The use of multi-wavelength images has provided the ability to objectively differentiate lymphocytes and granulocytes in culture based on endogenous contrast. We have started a preliminary pilot study is to investigate how multi-wavelength confocal images compare to those from previous generations of reflectance confocal microscopes that used a single wavelength (typically 820 nm). The following image characteristics will be studied in lesions and in normal skin: The difference in image brightness/scattering signal between images acquired sequentially using 785 nm, 815nm and 850nm laser light. In particular, we will look at the wavelength scattering differences of keratin versus melanin and of epithelium versus papillary collagen. We will seek to identify immune cells using wavelength dependent image intensity.

The primary outcome of the study will be a point estimate of the information differences that the multi-wavelength images provide in normal skin, pigmented lesions and non-pigmented lesions. The results will be used to establish the image processing algorithms that will aid in the clinical interpretation of in-vivo dermatology imaging and inform the power calculations for future studies of CSLM for in-vivo diagnosis of skin lesions.

Keywords: skin cancer, immune cells, *in-vivo* imaging

139 Targeting the Innate Immune System to Enhance Cancer Therapy

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The immune system functions through both the innate and acquired arm. Innate immune response is immediate, non-specific, and does not amplify with re-challenge of a specific challenge. In contrast, the acquired immune system involves T-cells, B-cells, and dendritic cells with slower response following initial pathogen exposure but that amplifies with re-challenge. Given the ability of the immune system to amplify response with subsequent re-challenge, much attempt has been made to enhance this component of the immune system through vaccines and immune stimulants as part of cancer therapy. Unfortunately, these efforts have not yielded major therapeutic advances to the treatment of cancer. Reasons for this include transformed tumors from solid tumors and hematopoietic malignancies are able to recruit multiple mechanisms to suppress the acquired immune system. Our translational program project focuses on therapeutic interventions to both enhance the innate immune system and augment other therapies dependent upon innate immune function. Project 1 focuses on development of small modular immune pharmaceuticals (SMIP) directed at CD37. CD37 SMIP is a peptide-based therapy targeted toward the B-cell specific CD37 antigen with an intact FcγR binding domain that mediates potent NK-cell mediated cytotoxicity and also direct cytotoxicity. This project also focuses on pre-clinical and clinical development of IL-21 in CLL and related lymphoid malignancies Project 2 focuses on FcγR function in monocytes and enhancing monocyte killing of tumor cells coated with therapeutic antibodies through the use of novel immune modulating agents. Project 3 examines NK cell activation through PP2A mediated signaling and the ability of TGF-β antibodies to enhance anti-tumor activity against a variety of solid tumor malignancies. Project 4 focuses on enhancing NK-cell mediated involvement in solid tumor cancer antibody therapeutics that recruit effector cell function. Agents to enhance NK cell function in this context include CpG oligonucleotides, IL-21, and TLR7 agonists. Each project interacts closely with the established cores (statistics, animal, administration) to assure integrated projects involving all of the members of this research team. Over the 7 years of funding provided by this grant several hundred patients have been enrolled in early phase I/II clinical trials derived from the research focus of the projects within this grant. At present, five clinical trials spanning six different types of cancer are actively being pursued in the context of innate immune based therapies.

Keywords: innate immune system, immunotherapy, antibody therapeutics

140 Human Monoclonal Antibodies Targeting Components of the IGF System and other Cancer-related Proteins

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The role of the IGF system for development of cancer has recently been validated as a promising target for cancer treatment. Currently, there are more than ten antibodies against the IGF-I receptor in Phase I/II clinical trials. We have hypothesized that targeting the ligands especially IGF-II could also inhibit signaling through the insulin receptor (IR), and may not require penetration into the tumor. We have developed several human monoclonal antibodies (hmAbs) against components of the IGF system, including IGF-II, IGF-I, and BP2. One of these antibodies, m610, which recognizes both the mature and the premature forms of IGF-II, inhibited the proliferation of the neuroblastoma cell lines tested. Because IGF-II secretion is up-regulated in many types of tumors, especially childhood malignancies such as neuroblastoma, osteosarcoma, and Ewing's sarcoma, therapy targeting ligands could be beneficial. This antibody is also able to deplete soluble IGF-II from serum and tissues, which blocks autocrine and paracrine mechanisms. It not only blocks signals through IGF-IR, but also blocks IGF-II binding to the insuling receptor (IR) and activation of IR. Another antibody, m708, is cross-reactive for both IGF-I and IGF-II. These antibodies, while already with nM affinity, are being further affinity matured. Two IGFBP2-specific antibodies have also been developed. The two antibodies bound to distinct epitopes on BP2. Interestingly, binding of these antibodies did not interfere with the formation of IGF-BP complex, which could enhance clearance of IGFs, both in free or complexed forms, from the circulation. Other antibodies are being characterized and new antibodies are being identified with a major goal to develop a large panel of different classes of antibodies that could affect the function of the IGF system by various mechanisms including blocking the ligand-receptor interactions, internalization of the receptor, inhibition or enhancement of signal transduction by alosteric effects, and mimicry of ligands or receptors, and evaluate their potential as cancer therapeutics. We have also identified and characterized to various extents antibodies against mesothelin and CD22 (in collaboration with Ira Pastan), and against DR4 and DR5.

Acknowledgment: Dr. Carol Thiele, Cell & Molecular Biology Section, Pediatric Oncology Branch, NCI Center for Cancer Reseach
Dr. Crystal Mackall
Dr. Lee Helman
Systems Medicine, Inc

Keywords: therapeutic antibodies, IGF, cancer

141 Preliminary Results of a Phase I Study Using Intravesical Administration of Adenoviral-Mediated Interferon-α for Patients With Transitional Cell Carcinoma of the Bladder

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Background: BCG is currently the gold standard for the treatment of recurrent superficial bladder cancer. Despite BCG and other second line therapies, recurrence is a significant problem. Intravesical gene therapy is under investigation as a viable treatment modality due to direct contact between vector and tumor, isolation from vital organs, and easy access to urine and tissue to monitor therapy effects.

Methods: A Phase I, non-randomized, dose escalating, two-center, open label intravesical gene therapy study for BCG refractory superficial bladder cancer patients with CIS/pTa or pT1 disease who refuse cystectomy. Intravesical administration of Ad-IFNα (75ml) with 5 dose levels ($3x10^9$, $1x10^{10}$, $3x10^{10}$, $1x10^{11}$, and 3×10^{11} particles/ml) is planned. 1mg/ml of Syn3 is used as an excipient. Pre-treatment, 0-24 hour, 24-48 hour and 1st voided urine specimens on days 3-7, 10, 14, 28 are tested for IFNα by ELISA. The primary endpoint is safety and tolerability. A secondary endpoint is IFN production detected in urine. The results from the first 11 patients will be presented.

Results: Initial urinary urgency and failure to retain the intravesical instillation for a full hour was present in the first two patients treated with Ad-IFNα/Syn3. Patients 3-11 were pretreated with anticholinergics prior to instillation with minor immediate and no prolonged urinary urgency. Urinary IFN levels (determined at MDACC) for patients receiving $3x10^9$ Ad-IFNα were below the lower limit of the assay (156pg/ml). Peak interferon levels for groups 2-4 were 1038, 10568, and 4689 pg/ml, respectively. Urinary interferon values remain elevated above baseline for 4-7 days. Three patients have had clinical responses and have received a second intravesical instillation.

Conclusions: Preliminary results demonstrate that intravesical administration of Ad-IFNα/Syn3 is safe and well tolerated. Dose levels of $1x10^{10}$ particles are able to initiate prolonged production of urinary IFNα. Continuing studies with higher doses will establish the safety and efficacy of this treatment.

Keywords: bladder cancer, gene therapy, interferon

142 City of Hope Lymphoma SPORE

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City of Hope/Beckman Research Institute

The overall goal of the City of Hope Lymphoma SPORE is to develop translational studies to improve the therapy of Hodgkin's and non-Hodgkin's lymphoma. This grant consists of five translational research projects to develop novel approaches that are derived from molecular and immunologic studies of T cell and antibody-based therapies. An important theme of the translational studies in this grant is to develop lymphoma therapies that will reduce toxicities associated with current treatment regimens for Hodgkin's and non-Hodgkin's lymphoma which can then be translated to the older patient population. The major goal of Project I is to develop anti-CD25 based radioimmunotherapy of lymphoma for Hodgkin's and CD25+ T cell lymphoma. Project II focuses on utilizing cellular immunotherapy for advanced B cell lymphoma utilizing engineered CD19-specific T cells. Investigators in this project have developed a T cell genetic modification platform for expressing chimeric immunoreceptors that redirect antigen specificity and effector function of T cells towards cell surface epitopes on lymphomas and will utilize chimeric antigen transduced T cells that will be expanded in vivo. Because epidemiologic studies indicate that stem cell damage from pretransplant therapeutic exposures may play a role in the development of myelodysplasia, Project III is conducting longitudinal study of a population of patients with Hodgkin's and non-Hodgkin's lymphoma to investigate the cellular and molecular factors that are predictive for development of myelodysplasia and to determine the molecular sequence of events that lead to myelodysplasia. In Project IV, investigators have developed molecularly engineered constructs for anti-CD20 directed therapeutics to improve imaging, radioimmunotherapy and novel immunocytokine (anti-CD20 antibody–IL-2) for the treatment of patients with CD20+ lymphoma. An important component of this project will be to delineate the immunologic effector mechanisms operative in immunocytokine-mediated anti-lymphoma in vivo activity. In Project V, investigators at City of Hope and Caltech are developing siRNA-based therapeutics in lymphoma utilizing an antibody-based Cyclodextrin (CDP) polymer system to deliver siRNA to targeted specific genes in lymphoma pathogenesis and clinical behavior.

Keywords: radioimmunotherapy, adoptive T cell therapy, myelodysplasia, siRNA

143 Late Stage Clinical Development of Gene Therapy Approach for Prostate Cancer

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Henry Ford Health System

For the past 15 years our research program has been developing a gene therapy-based approach to treat cancer. Our approach utilizes an oncolytic, replication-competent adenovirus to deliver a pair of therapeutic suicide genes to tumors. The oncolytic adenovirus replicates in, and selectively kills, malignant cells. The suicide gene therapy provides a local chemotherapeutic effect and sensitizes tumor cells to ionizing radiation.

We have successfully translated our approach into the clinic resulting in 6 clinical trials in prostate and pancreatic cancer, including a recently opened randomized, controlled phase 2/3 trial in newly-diagnosed, intermediate-risk prostate cancer. A second randomized, controlled phase 2/3 trial in locally recurrent prostate cancer is being planned for 2008. Our research team, which is comprised of about 12 people (scientists, physicians, technical/clinical staff), conducts all phases of product development including preclinical efficacy and toxicology testing of our products, preparation and submission of required documents (INDs, protocols) to federal agencies (FDA, NIH/RAC) and institutional committees (IRB and IBC), and execution of our clinical trials. During the past 10 years, we have sponsored 4 Investigational New Drug (IND) applications to the FDA, and we currently have 2 products in the clinic.

Our work has been supported almost entirely by NCI-sponsored investigator-initiated grants, including an active Program Project Grant (P01) entitled “Molecular Gene and Radiation Therapies for Cancer”. The current P01 grant supports two preclinical projects, one clinical project that proposed three phase 1/2 clinical trials, and four cores. The projects and cores interact synergistically, making the whole greater than the sum of its parts. The vast majority of the proposed work has been completed and has now progressed into late stage clinical development. Our randomized, controlled phase 2/3 trials contain both clinical and molecular endpoints, the latter of which are designed to test specific hypotheses regarding the molecular basis for the previously observed clinical activity. We hope our clinical trials demonstrate “proof of concept” and generate new knowledge that will foster the development of future products.

Keywords: gene therapy, radiation therapy, clinical trials

144 Antibody-Targeted Therapeutics for B-cell Malignancies: From Bench to Clinic

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A variety of antibody-based therapeutics have been developed, with the greatest clinical impact being in the treatment of hematopoietic tumors, particularly B-cell types. We have focused on developing humanized monoclonal antibodies (MAbs) for use alone or as conjugates with radionuclides, cytokines or drugs, such against as CD20, CD22, CD74, HLA-DR, MUC1, and CEACAM6.

Our goal is to examine how these antibodies or antibody-conjugates can be best used to treat non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM), proceeding from cell culture, xenografts models, Cynomolgus monkeys, to patients, collaborating with 2 biotechnology firms and involving 4 academic research institutions, and involving discovery, preclinical testing, antibody engineering, GMP manufacturing, and Phase I/II clinical trials. Over the past 3 years of PO1 funding from the NCI for preclinical development, we have advanced 2 novel therapeutic agents to Phase-I or Phase- I/II clinical testing (veltuzumab, humanized anti-CD20 IgG in NHL), milatuzumab (humanized anti-CD74 in NHL, MM and CLL), as well as developing a novel CD20-bispecific antibody system for pretargeting radionuclides for both imaging (ImmunoSPECT and ImmunoPET) and therapeutic uses. This latter system is currently under the initial stages of product development and manufacturing for introduction into the clinic in the future, following encouraging preclinical results (Sharkey et al., Cancer Res. 2008 Jul 1;68(13):5282-90), especially in combination with anti-CD20 MAb (veltuzumab) immunotherapy, based on preclinical findings showing an efficacy advantage for this combination (Mattes et al., Clin Cancer Res, in press).

Encouraging preclinical results with an anti-CD20 MAb-intereron-2 b conjugate also justifies translating these findings to patients. The Dock-and-Lock (DNL) technology being developed for constructing multivalent, multifunctional antibodies is also being applied to the development of pretargeted immunoSPECT and immunoPET with various candidate targets of NHL and MM in order to improve specific targeting and imaging, to be compared to FDG-PET in these indications, especially for assessing therapeutic response and minimal residual disease. Other projects include DNL-engineered hexavalent anti-CD20 and anti-CD20/CD22 bifunctional constructs, studying both mechanisms of action in comparison to parental bivalent MAbs as well as their candidacy for clinical trials. Thus, these projects combine several unique antibody constructs in a multidisciplinary, highly translational, well-integrated, program that rapidly transfers new agents from the laboratory to the clinic with the goal of improving the therapeutic prospects in 3 B-cell malignancies. (Supported in part by NCI grants P01-CA103985 and R21-CA126060 from the National Institutes of Health.)

Keywords: non-Hodgkins lymphoma, immunotherapy, radioimmunotherapy

145 Preclinical Studies and Clinical Experience With the Combination of Interleukin-12 with Trastuzumab and Paclitaxel in HER2-overexpressing Malignancies

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Natural killer (NK) cells express an activating receptor for the Fc portion of IgG (FcγRIIIa) that mediates interferon (IFN)-γ production in response to antibody (Ab)-coated tumor cells. The anti-tumor effects of trastuzumab, a humanized monoclonal antibody (mAb) that binds the extracellular domain of HER2, may depend on the expression of Fc receptors (FcR). We have previously demonstrated that NK cells activated with interleukin (IL)-12 in the presence of immobilized IgG secrete >10-fold higher levels of IFN-γ as compared to stimulation with either agent alone. We therefore hypothesized that the combination of tumor specific Ab and a cytokine would elicit superior anti-tumor activity in vivo. A murine colon adenocarcinoma engineered to express human HER2/neu ($CT\text{-}26^{HER2neu}$) was employed to test this hypothesis. Tumor bearing BALB/c mice were treated with PBS, muIL-12, anti-HER2 mAb (4D5), or the combination. The combination of IL-12 and 4D5 significantly reduced tumor growth ($p<0.0001$) and this was associated with elevated levels of circulating IFN-γ and chemokines (IFN-γ, RANTES, IL-8, TNF-α and MIP-1α). The anti-tumor effects of this treatment combination were abrogated in IFN-γ-deficient mice and mice depleted of NK cells via anti-asialo GM1 Ab.

These pre-clinical data suggested trastuzumab and interleukin-12 (IL-12) could synergistically stimulate NK cell cytokine secretion and cytotoxicity. Given the widespread use of trastuzumab with cytotoxic agents, we conducted a phase I trial that employed IL-12 in combination with trastuzumab and Paclitaxel (NCI 84). Trastuzumab was given on day 1 of the weekly cycle in combination with i.v. injections of IL-12 on days 2 and 5 starting in week 4. Paclitaxel was given every 3 weeks. This trial accrued 21 patients with metastatic 2+ and 3+ HER2-positive tumors. The average age of patients enrolled in this trial was 57.7 years. We observed one complete response (CR), four partial responses (PR), and stabilization of disease (SD) occurred in 6 patients. Four of five clinical responses occurred in patients with HER2 3+ disease. Correlative studies demonstrated significantly increased serum IFN-γ and activation of the MAPK and ERK in peripheral blood mononuclear cells from patients with clinical benefit. No patients with progressive disease had activation of ERK or measurable levels of serum IFN-γ. These data are in accordance with our previous phase I trial of trastuzumab with IL-12. We have now examined the intracellular signaling pathways responsible for synergistic IFN-γ production by NK cells in response to IL-12 and FcR stimulation. Synergistic IFN-γ production was dependent on co-localization of IL-12R and FcγRIIIa within lipid rafts which in turn led to activation of ERK and Syk.

The modality being developed is useful as a combination therapeutic agent. Our data provide in vitro and in vivo evidence that tumor regression in response to regimen with Trastuzumab and IL-12 is dependent upon the secretion of IFN-γ by NK cells. Our data further suggest that the clinical effects of anti-tumor mAbs can be enhanced through co-administration of NK cell activating cytokines. The addition of IL-12 to regimens containing trastuzumab and paclitaxel may lead to enhanced clinical efficacy through the induction of anti-tumor immunity. Dual recruitment of FcγRIIIa and the IL-12R to lipid raft micro-domains allows for enhanced activation of downstream signaling events that lead to IFN-γ production.

Keywords: interleukin-12, trastuzumab, interferon-gamma

146 Activation of Plasmacytoid Dendritic Cells With TLR Agonists in Order to Induce Improved Antitumor Immune Responses Against Melanoma

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Although cancer vaccines offer significant promise, clinical effectiveness has yet to be fully realized. Effective vaccines may require inclusion of basic elements of the immune response which allow successful elimination of pathogens. It is now clear that strong adaptive immune responses are preceded by a potent innate immune response, triggered by pathogen-associated molecular patterns (PAMPs) that are recognized by immune cells expressing Toll-like receptors (TLRs). In the absence of TLR signaling, release of inflammatory cytokines by innate immune cells is sub-optimal. Tumor cells typically do not trigger TLR signaling and this contributes to the lack of effective antigen-specific immunity in solid tumors. Harnessing and adapting the mechanisms used by pathogens to induce effective immunity represents a promising approach to improving antigen-specific antitumor immune responses. The plasmacytoid dendritic cell, the primary producer of type I interferons in the body, is a central mediator of anti-viral innate immunity and coordinates immune cell interactions which lead to a potent adaptive T-cell response. This innate immune response is important not only to trigger strong T-cell priming, but also to induce inflammation at the target site which leads to enhanced T-cell migration and effector function. In our murine models, we have found that plasmacytoid dendritic (pDC) cells can lead to enhanced antigen-specific T-cell immune responses, partially through synergy with myeloid dendritic cells (mDC) and activation of NK cells. In addition, we and others have found that pDC can be activated directly in vivo through specific TLR ligands. We will now test these concepts in melanoma patients and will utilize a vaccine in combination with a TLR agonist capable of activating both pDC and mDC in order to model the synergy observed in our murine system. We will measure T-cell priming in patients immunized in the presence or absence of TLR activation. Subsequent to T-cell priming, we will administer a TLR agonist at the tumor site in order to induce inflammation. We will test the ability of this intervention to activate pDC and mDC at the tumor site and enhance T-cell migration into the tumor and T-cell effector function. These studies may lead to the development of improved immunotherapeutic treatment regimens against melanoma as well as other common cancers.

Keywords: melanoma, cancer vaccines, dendritic cells and T-cells

147 Particle-Based MonoClonal Antibody Detection in Serum for Optimal Drug Dosing

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Monoclonal antibodies are used in the treatment of many cancers and proliferative diseases. Several approaches have been used to study the pharmacokinetics of these treatments. The target molecule can be made recombinantly for sandwich ELISA assays. However, it may not always be possible to generate the recognized portion of the target molecule and it is expensive and cumbersome to generate large amounts of recombinant protein. An alternate approach is to express the target molecule on a cell line by transfection, using flow cytometry to assess the binding of the desired mAb. This method has been used for alemtuzumab (anti-CD52), but is difficult to develop, requires skilled personnel to execute, and has limited sensitivity. Finally, ELISA assays have been developed that used antibodies specific for the therapeutic mAb. The antibodies used for this purpose are either anti-idiotypic or specific for residual non-human sequences of the therapeutic mAb, as was the case with alemtuzumab. However, each of these approaches is technically demanding and has limited sensitivity when used in biologic samples because of high background.

We selected peptide sequences recognized by alemtuzumab (anti-CD52) or rituximab (anti-CD20) from phage displayed peptide libraries. Synthetic biotinylated peptides were used in an enzyme linked immunoadsorbant assay (ELISA) and had a sensitivity of less than 0.05μg/ml in saline buffer, but the functional sensitivity in serum was limited to ~1ug/ml by the need to dilute samples to reduce background. Therefore, we developed a complementary immunoassay in which the peptides were synthesized on the surface of 10μm diameter tentagel beads. mAb binding was detected by fluorochrome labeled secondary antibodies via flow cytometry. There was negligible background signal on the beads, even in neat serum. The functional sensitivity using peptide-beads was less than 0.05μg/ml. The enhanced sensitivity of the bead based assay is ideal for detecting very low levels of the target antibody, while the conventional ELISA is sufficient when the target antibody concentrations present at concentrations of >1.0ug/ml. The process outlined here is generalizable to any mAb and could enable improved pharmacokinetic analysis during development and clinical use of these therapies. The clinical goal would be to regulate mAb dosage for each patient based on concentration in the blood instead of dosing based on body weigh in order to deliver an optimal dose to the patient and minimize the cost to the health care system.

Keywords: pharmacokinetics, personalized medicine, monoclonal antibodies

148 A Phase I Study of De-Immunized DI-Leu16-IL2 Immunocytokine in Patients With B-cell Non-Hodgkin's Lymphoma

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CD20 has been an attractive molecule to target for the immunotherapy of Non-Hodgkin's Lymphoma. Recent clinical work has indicated that the activity of Rituxan can be increased by the administration of concomitant immunocytokines; the first was a study with interleukin-2 and the second was a recent study with GM-CSF. Several years ago our group began the engineering of a fusion protein, combining a humanized anti-CD20 antibody based on Leu-16 and the IL2 molecule. We have shown the activity of this immunocytokine in animal models and have published the results. Over the past years we have undertaken the production of clinical grade material and have now obtained an IND from the FDA and have recently begun clinical trials with this novel reagent.

The initial clinical trial was constructed as a standard Phase I dose escalation study for patients with relapsed CD20 positive lymphoma post autologous hematopoietic stem cell transplant for all histologies except high grade lymphomas. The design was constructed with co-administration of Rituxan, using doses of Rituxan to deplete B cells and maintain circulating levels greater than 5 microgram/ml. Following the Rituxan, the patients were given two doses of Anti-CD20-IL2 separated by one day, with 4 hour infusions. Patients were also given concomitant Indocin, starting before the Anti-CD20-IL2 and continuing through the immunocytokine infusion. Comprehensive monitoring was conducted including physiologic parameters, Rituxan levels, B cell levels, extensive immunophenotyping, and cytokine levels.

Two patients have now been treated on the protocol. The B cell levels were measured utilizing both CD19 and CD20. Rituxan levels were adjusted based on previous pharmacokinetic models from earlier studies with Rituxan. The doses of administered Rituxan varied from 50mgs/square meter to 20 mgs/square meter. The administered doses cleared the circulating B cells as expected and documented by repeat testing. The initial dose of the immunocytokine was 0.5mgs/square meter BSA. We were surprised to observe immunologic activation at this low dose, confirming the activity we had seen in animal models. The patients had transient rises in LDH with mild weight gain. The cytokine profiles were most notable in IL2, IL6, IL2R. IP10, and MIG levels.

The first patient has remained with stable disease during treatment and has had an improvement in platelet count from 50K to 100K. He also had return of allergy to shrimp, manifested by a skin rash to shrimp which had not occurred since he initiated chemotherapy for his lymphoma many years prior to his transplant. The second patient has just completed therapy and we are in the process of evaluating his disease status. Based on the immunocytokine pharmacokinetics and pharmacodynamics as ascertained from the cell populations appearing in the peripheral blood, plans are now underway to modify the protocol to optimize the immunotherapeutic indices.

Keywords: lymphoma, immunocytokine, CD-20

149 Phase I Study of Intraperitoneal Denileukin Diftitox (ONTAK) in Patients With Advanced Ovarian Cancer

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Background: CD4+CD25+Foxp3+ regulatory T cells (Tregs) which induce T cell suppression are increased in the ascites and peripheral blood of ovarian cancer patients and associated with a poor prognosis. ONTAK, a diphtheria/IL-2R fusion protein depletes peripheral blood Tregs in humans when given IV and induces an anti-tumor response in mice. We hypothesized that intraperitoneal (IP) infusion of ONTAK may reverse immune suppression in the peritoneum by depleting local Tregs. A phase I dose escalation study was initiated to establish the maximally tolerated dose (MTD) of IP ONTAK, and assess its effect on Tregs in patients with ovarian cancer.

Methods: Up to 18 subjects with refractory ovarian cancer are enrolled to 1 of 3 Arms defined by dose level. Arm 1 (5µg/kg) is enrolled first, then Arm 2 (15µg/kg), then Arm 3 (25µg/kg). A treatment cycle consists of IP ONTAK on Days 1-3 every 14 days for a total of 4 cycles. The primary endpoint is to establish the IP MTD. Secondary endpoints include effect of ONTAK on Tregs and cytokines in the peripheral blood and ascites, and clinical activity. Toxicity is evaluated on Days 1-3, 8, and 14 of each cycle. Tregs are assessed using RT-PCR and cytokines using LUMINEX at baseline, and after cycles 2 and 4. CA125 is checked serially during ONTAK treatment.

Results: Nine subjects have been enrolled, three to Arm 1 and six to Arm 2. Median age is 56 years (range, 34-73) and median number of salvage chemotherapy regimens is 3 (range, 2-7). Three subjects in Arm 1 and 4 of 6 subjects in Arm 2 have completed 4 cycles without dose-limiting toxicity (DLT). In Arm 2, 1 subject had DLT (Grade 3 esophagitis) and 1 had disease progression after cycle 1. Toxicity in both Arms was primarily Grade 1 and 2. Tregs (reported as relative quantitation level of Foxp3 normalized to CD4) in peripheral blood and ascites decreased in 1 of 3 subjects in Arm 1 and 3 of 4 subjects in Arm 2. CA125 decreased in 4 of 6 subjects in Arm 2 by a median value of 92 (range, 26-200). Tregs analyses for subjects in Arm 2 and cytokine analyses for all subjects are on-going.

Conclusions: Data suggests that IP ONTAK at 5-15 mcg/kg is well-tolerated in subjects with refractory ovarian cancer; and results in decreased CA125 and decreased peripheral blood and ascites Tregs. Accrual to Arm 3 continues.

Keywords: ovarian cancer, regulatory T cells, intraperitoneal therapy

150 Overcoming Tumor Antigen Anergy in Human Malignancies Using the Novel IDO Enzyme Inhibitor, 1-Methyl-D-Tryptophan

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Background: Many of the limited responses seen in cancer therapeutic trials using immunotherapy appear to be in part due to the ability of tumors to promote host immune tolerance to their own antigens through various mechanisms. One such pro-toleragenic mechanism that has gained much attention is through the action of the heme containing enzyme indoleamine 2,3-dioxygenase (IDO) encoded by the INDO gene. IDO is expressed by both malignant and normal cells in an inducible fashion upon exposure to γ-interferon. It is the rate limiting initial step in the breakdown of the essential amino acid tryptophan into various active metabolites such as kynurenine and NAD+. The role of IDO in modulating the immune response was first elucidated by the work of Andrew Mellor and David Munn in 1998 showing it prevented rejection of the fetus in pregnant mice. Initial studies in Lewis lung cancer (LLC) tumor bearing mice performed by Antonia and Munn suggested IDO expressing mononuclear cells in tumor draining lymph nodes played a role in mitigating the anti-tumor immune response. Preclinical data support the activity of 1-methyl-D-tryptophan (1-MT, an IDO inhibitor) in preventing T cell anergy in tumor draining lymph nodes, delaying growth of transplanted LLC mouse xenografts, and working synergistically with various chemotherapeutic agents in regression of autochthonous breast tumors in MMTV-Neu mice.

Through collaboration with the NCI RAID program and CTEP, and support from the Southeast Phase II Consortium (SEP2C), a phase I trial of 1-MT was initiated with support from the NO1 CM66208 contract. The SEP2C is a relatively new N01 supported consortium consisting of the Moffitt Cancer Center, Massey Cancer Center, Vanderbilt-Ingram Cancer Center, University of North Carolina Lineberger Cancer Center, and the Emory Winship Cancer Center.

Study Design and Current Status: The trial is a phase I single arm, toxicity and safety, first in man trial using a conventional 3+3 dose escalation design. The patient population is subjects with solid refractory malignancies age 18 or older with PS 0-1. Additional criteria are no active autoimmune disease, no second active malignancies, and intact organ function. The planned accrual goal is 25 patients. Proposed correlative studies include assessment of IDO expression in solid tissue biopsies using immunohistochemistry, kynurenine/tryptophan ratios using HPLC and MS at baseline and during treatment, and quantitative changes in circulating Treg cells using CD4/CD25 flow cytometry with FoxP3 staining. As of 7/15/08, three patients have been treated on study. The first patient with metastatic GE junction cancer was replaced after suffering a CVA two weeks into the trial which was not related to the study drug. The second patient with ovarian cancer was treated for six weeks on study when she developed hypophysitis. Her response scans showed stable disease, but was not retreated with study drug after treatment for hypopituitarism. She died of progressive disease two months after coming off study. A third patient with metastatic melanoma is currently on treatment with 1-MT but has not reached the first evaluation interval. Of note, all three patients demonstrated marked increases in their CRP levels within the first week of treatment with 1-MT. Accrual to the trial continues.

Keywords: immunotherapy, immune tolerance, 1-methyl-D-tryptophan

151 Activating Endogenous Mechanisms That Enhance Anti-Tumor Immune Responses

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Fighting disease by harnessing endogenous, natural, defense mechanisms in conjunction with pharmacological and surgical interventions is likely to be clinically beneficial. The fight-or-flight stress response is one of nature's under-appreciated defense mechanisms that activates multiple psycho-physiological systems to promote survival. We have proposed that it may be useful to identify biological mechanisms by which a fight-or-flight stress response promotes survival because these mechanisms could be clinically harnessed to fight disease. Stress can be defined as a constellation of events that begins with a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiologic fight/flight systems in the body (stress response). The idea that a stress response may be harnessed to fight disease seems counter-intuitive at first glance because numerous studies have shown that chronic stress/distress can suppress or dysregulate immune function (Nat Rev Immunol, 2005). For example, studies conducted in our laboratory have shown that chronic stress suppresses cell-mediated immunity, enhances regulatory T cell numbers, and increases susceptibility to ultraviolet-B (UVB)-induced squamous cell carcinoma (SCC) (Brain, Behavior, Immunity 1997; J Nat Cancer Inst, 2005).

However, in contrast to chronic (weeks to months) stress, an acute (minutes to hours) fight-or-flight stress response is one of nature's fundamental psycho-physiological survival mechanisms (e.g. without this response, a lion has little chance of catching a gazelle, and a gazelle has little chance of escape). We hypothesized just as the acute stress response prepares the brain, heart, and muscles for fight or flight, it may also prepare the immune system for challenges (e.g. wounding or infection) that may be imposed by a stressor (e.g. predator). This hypothesis was confirmed by studies showing that acute stress experienced at the time of immune activation or antigen exposure induces a redistribution of circulating immune cells, increases leukocyte trafficking to sites of wounding or immune activation, enhances innate and adaptive immunity, and induces a long-lasting enhancement of cell-mediated immunity (J. Immunol 1995, 1996; PNAS, 2000, 2005; Amer J Physiol, 2005; Int Immunol, 2005). Based on these studies, we set out to examine the effects of acute stress-induced immuno-enhancement in the context of cancer. Because SCC is an immuno-responsive cancer, we hypothesized that acute stress experienced immediately prior to UVB exposure, would enhance cell-mediated immunity and increase resistance to SCC. To test this hypothesis, mice were exposed to UVB (minimum erythemal dose, 3-times/week, weeks 1-10). The control and acute stress groups were treated identically except that the stress group was restrained for 2.5 h before each of nine UV sessions during weeks 4 to 6. Tumors were measured weekly, and tissue collected at weeks 7, 20, & 32. Chemokine and cytokine gene expression was measured by quantitative PCR, and CD4+ and CD8+ T cells were enumerated by immunohistochemistry and flow cytometry. Mice that were acutely stressed showed greater cutaneous T cell attracting chemokine (CTACK)/CCL27, RANTES, IL-12, and IFN-γ gene expression (at weeks 7, 20, & 32), higher T cell numbers in skin (weeks 7 & 20) and blood (week 20), and fewer tumors (weeks 12 to 19).

These results suggest that activation of acute stress physiology increases leukocyte trafficking and/or function during/following UV exposure, and produces a long-lasting enhancement of Type 1 cytokine-driven cell-mediated immunity that is crucial for resistance to SCC. These findings begin to identify psycho-physiological mechanisms that may be harnessed through pharmacological and/or bio-behavioral interventions to enhance immune system mediated elimination of tumors that are either naturally immunogenic, or rendered immuno-responsive via tumor immunotherapy.

Keywords: adjuvant immune-enhancement, psychological stress, skin cancer, squamous cell carcinoma

152 IL-15 in the Treatment of Metastatic Malignant Melanoma or Metastatic Renal Cell Carcinoma

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Interleukin-2 (IL-2) and interleukin-15 (IL-15) have pivotal roles in the control of the life and death of lymphocytes. Although their heterotrimeric receptors have two receptor subunits in common, these two cytokines have contrasting roles in adaptive immune responses. The unique role of IL-2 is the elimination of self-reactive T cells to prevent autoimmunity. In contrast IL-15 favors the survival of NK and CD8-memory T cells and is thus directed to the persistence of an immune response. IL-15 and IL-15R alpha are co-expressed on activated antigen-presenting cells. IL-15R alpha recycles and presents bound IL-15 *in trans* as part of an immunological synapse with neighboring NK and memory phenotype T cells. In light of their functional and differences in the life and death of lymphocytes, IL-15 may be superior to IL-2 in the therapy of cancer. A number of studies in murine models have suggested that IL-15 may prove of value in the therapy of neoplasia. For example, wild type B-6 mice following intravenous injection of MC38 syngenic colon carcinoma cells died within 6 weeks of pulmonary metastases. In contrast to IL-15 transgenic mice survived for more than 8 months following the infusion of tumor cells. In other studies IL-15 therapy prolonged the survival of mice that received syngeneic colon carcinoma CT26 cells intravenously. IL-15 was also active in murine melanoma models. In parallel studies that were designed to take advantage of the role of IL-15 in the generation and maintenance of memory-T cells, the gene that encodes IL-15 was incorporated into molecular vaccines. When administered to mice vaccinia vectors co-expressing IL-15 and an HIV antigen but not those expressing IL-2 instead of IL-15 induced long-lasting cellular immunity for the HIV antigen. On the basis of these studies my research group (in conjunction with the Biopharmaceutical Development Program (BDP), National Cancer Institute, Bethesda, Maryland) has produced recombinant human IL-15 (rhIL-15) under current good Manufacturing Practices (cGMP) conditions for use instead of IL-2 in human clinical trials. These trials will involve patients with metastatic renal cancer or malignant melanoma. Furthermore in light of the crucial role of IL-15 in the generation and maintenance of high avidity CD8+ memory T cells, molecular vaccines directed toward HIV and cancer antigens incorporating the gene encoding IL-15 have been produced and tested in animal models to provide a new perspective for the development of preventive vaccines against cancer associated pathogens, HIV and other infectious agents.

Keywords: IL-15, adaptive immunity, memory T-cells

153 Tumor Immunobiological Differences in Prostate Cancer Between African-American and European-American Patients

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Incidence and mortality rates of prostate cancer vary substantially amongst ethnic groups. Most notably, African-American men have the highest risk of developing prostate cancer, and due to the manifestation of a more aggressive disease, they have over twice the mortality rate of European-American men. While socioeconomic factors contribute to this health disparity, they do not fully explain the differences in prostate cancer incidence, aggressiveness, and mortality amongst the various ethnic groups in the United States.

We tested the hypothesis that differences in tumor biology contribute to this survival health disparity between African-American and European-American patients. Using microarray technology, we obtained gene expression profiles of primary prostate tumors resected from 33 African-American and 36 European-American patients. These tumors were matched on clinical parameters. We also evaluated 18 non-tumor prostate tissues from 7 African-American and 11 European-American patients. The resulting datasets were analyzed for expression differences on the gene and pathway level comparing African-American with European-American patients. Our analysis revealed a significant number of genes to be differently expressed between the two patient groups. Using a disease association analysis, we identified a common relationship of these transcripts with autoimmunity and inflammation. These findings were corroborated on the pathway level with numerous differently expressed genes clustering in immune response, stress response, cytokine signaling, and chemotaxis pathways. Several known metastasis-promoting genes were also more highly expressed in tumors from African-Americans. Furthermore, a two-gene tumor signature was identified that accurately differentiated between African-American and European-American patients.

In conclusion, the gene expression profiles of prostate tumors indicate prominent differences in tumor immunobiology between African-American and European-American men. The profiles portray the existence of a distinct tumor microenvironment in these two patient groups. Although preliminary, these findings are novel and could have implications for cancer therapy. Clinical trials of immunotherapy for prostate cancer have been conducted, and these therapies may enter clinical practice in the near future. Our data suggest that African-American patients and European-American patients might respond differently to these types of therapy.

Keywords: prostate cancer, cancer health disparity, gene expression profiles

154 Initial Rituximab Infusion Induces Rapid NK Activation that Persists after Resolution of Infusion Reactions

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Rituximab (R) monoclonal antibody is now a standard component of therapy for B-cell malignancies. Growing evidence indicates NK cells play a key role in the anti-tumor activity of R. For example, our *in vitro* studies demonstrate R-coated B cells activate NK cells as indicated by production of IFNg and phenotypic changes in NK cells including down-modulation of CD16 and upregulation of CD69 and CD54. Many patients experience infusion reactions during the initial hour of the first infusion with R. The mechanisms responsible for infusion reactions, and the relationship between these mechanisms and those responsible for the anti-lymphoma activity of R, are unclear. In order to study the behavior of NK cells during initial infusion with R, we evaluated complete blood counts, NK cell numbers, and phenotype in lymphoma subjects at various time points during the first infusion of R.

Subjects receiving a first dose of R (no R in the prior 12 months) for B cell malignancies were eligible. Blood samples were obtained prior to therapy, 30 minutes after the initiation of therapy (selected to coincide with typical time of infusion reaction), and 4 hours after initiation of therapy.

There was considerable inter-patient variability. Nevertheless, consistent patterns emerged across subjects. At the 30-minute time point, there was a drop in total white blood cell count and platelet count. These changes largely resolved by the 4-hour time point. There was also a drop in NK cell count at the 30-minute time point. However, in contrast to changes in the total white blood cell and platelet count, the decrease in NK cell numbers was persistent and remained at the end of the infusion. Phenotypic evidence for NK cell activation, including downregulation of CD16 and upregulation of CD54 and CD69, was seen at 30 minutes and increased progressively, so was most notable at the end of the infusion.

We conclude that, during R infusion, a non-specific drop in white blood cells and platelets roughly correlates temporally with infusion reactions. In contrast, NK cell activation and trafficking of NK cells out of the peripheral blood lasts beyond the time of clinical infusion reactions. Ongoing studies are exploring correlation between NK cell activation and cytokine production, complement fixation, and CD16 polymorphisms. These results could have implications related to not only the causes of infusion reactions, but also the role of NK cells in the anti-tumor activity of R.

Keywords: monoclonal antibody, lymphoma, NK cell

155 Pretreatment With Cisplatin Enhances Antigen-Specific CD8+ T Cell-Mediated Antitumor Immunity Induced by Therapeutic DNA Vaccination

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There is an urgent need to develop new and innovative therapies for the control of cervical cancer. Antigen-specific immunotherapy, such as DNA vaccines targeting human papillomavirus (HPV) oncoproteins E6 and E7, has emerged as a potentially plausible approach for the control of cervical cancer because these proteins are unique to, and constantly expressed in cervical cancer and are responsible for the malignant phenotype. We have developed a strategy to enhance DNA vaccine potency by generating a DNA construct encoding the molecular adjuvant, calreticulin (CRT) linked to HPV-16 E7 antigen (CRT/E7) (Cheng et al., 2001 JCI). Since the combination of multiple modalities for cancer treatment is more likely to generate more potent therapeutic effects for the control of cancer, we have explored the combination of chemotherapy using cisplatin, which is routinely used in chemotherapy for advanced cervical cancer, with CRT/E7 DNA vaccination in a preclinical model. Treatment with cisplatin may also boost immune responses by lysis of tumor cells. We characterized the combination of cisplatin with CRT/E7 DNA vaccine using different regimens for its potential ability to generate E7-specific CD8+ T cell immune responses as well as antitumor effects against E7-expressing tumors. Our results indicate that treatment of tumor bearing mice with chemo-immunotherapy combining cisplatin followed by CRT/E7 DNA generated the highest E7-specific CD8+ T cell immune response, produced the greatest anti-tumor effects and long-term survival and generated significant levels of E7-specific tumor infiltrating lymphocytes compared to all the other treatment regimens. Furthermore, we found that treatment with cisplatin enhanced the T cell-mediated lysis of E7-expressing tumor cells *in vitro* and increased number of E7-specific CD8+ T cell precursors in tumor bearing mice. In addition, we observed that more E7-specific $CD8^+$ T cells migrate to and proliferate in the location of TC-1 tumors in mice treated with cisplatin compared to untreated mice. Thus, our data suggest that chemo-immunotherapy using cisplatin followed by CRT/E7 DNA vaccine is an effective treatment against E7-expressing tumors in animal models and may potentially be translated into the clinical arena.

Reference: C.W. Tseng et al. 2008, Clinical Cancer Research 14: 3193-3203

Keywords: cisplatin, DNA vaccine, human papillomavirus (HPV)

156 Targeting Epidermal Growth Factor Receptor Signaling to Selectively Enhance Tumor Response to Radiation

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Epidermal Growth Factor Receptor (EGFR) is a 170-kDa transmembrane receptor tyrosine kinase (RTK) of the ERBB family. Previous work showed that many carcinomas express high level of EGFR and such overexpression was associated with poor prognosis.

The impetus for our translational research program leading to establishment of a new frontline non-surgical treatment for locally advanced head and neck squamous cell carcinoma (HNSCC) stemmed from our initial laboratory finding showing that murine tumors expressing higher levels of EGFR were more resistant to radiation. This observation was corroborated by a clinical correlative analysis using pretreatment tumor samples of patients with locally advanced head and neck cancer enrolled into a phase III radiotherapy trial, which demonstrated that higher tumor EGFR expression was highly significantly associated with higher local-regional tumor relapse and poorer survival rates after radiotherapy. We subsequently showed a causal relationship between EGFR expression and radiation resistance by revealing that transfection of a low EGFR-expressing tumor with human EGFR gene increased radioresistance. A follow up mechanistic oriented study showed that radiation-induced EGFR activation and the subsequent nuclear translocation amplified repair of DNA double-strand breaks by binding of repair proteins (DNA-PK, Ku70, and Ku80). Concurrently, through intramural multidisciplinary collaboration, we also showed that combining cetuximab to radiotherapy markedly enhanced the response of several human xenografts to radiotherapy using both regrowth delay and local tumor cure endpoints. These results along with the findings of two other laboratories contributed to the enthusiasm for rapid enrollment of patients into a phase III clinical trial for patients with locally advanced head and neck carcinoma. This pivotal study showed that the addition of only eight doses of cetuximab given during radiotherapy resulted in a significant improvement of the local-regional control (3-year rate: 47% vs 34%, p=0.005) without increasing radiation-induced morbidity.[7] The improvement in local-regional control rate translated to a significant increase (p=0.03) in the median survival time (from 29 to 49 months) and the 3-year survival rate (from 45% to 56%). This magnitude of improvements has not been achieved by combining cetuximab or other small molecule tyrosine kinase inhibitors with chemotherapy in solid tumors. Consequently, cetuximab was approved by the FDA for front line use with radiotherapy for the treatment of locally advanced head and neck carcinoma.

In summary, these series of integrated studies conducted over a span of nine years established the proof of principle that modulating a signaling pathway that is differentially expressed between tumors and normal tissues can lead to selective sensitization of tumors to radiotherapy and thereby improving the therapeutic ratio. Such selective enhancement of tumor response has not been observed by combining radiation with traditional chemotherapeutic agents. Building on this successful translational research, our group has expanded efforts to developing rational combinations of radiotherapy with other novel agents with or without chemotherapy in patients with locally advanced upper aerodigestive cancers.

Supported by grants P01-CA06294 and R01-CA84415.

Selected references: Milas et al., Clin Cancer Res *6:* 701, 2000; Ang et al., Cancer Res *62:* 7350, 2002; Dittmann et al., JBC *280:*31182, 2005; Bonner et al., N Engl J Med *354:* 567-578, 2006

Keywords: EGFR, radiation, head and neck carcinoma, DNA repair

157 Multi-Criteria Optimization of Intensity-Modulated Radiation Therapy

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We translate methods from socio-economy to radiation therapy. An important concept in socio economy, which is used in general multi-objective optimization, is Pareto optimality. Applied to cancer treatment, Pareto optimality characterizes a treatment in which we cannot improve the eradication of tumor cells without increasing the likelihood of side effects in one particular organ. Similarly, a Pareto-optimal treatment is one in which we cannot improve the sparing (in terms of possible side effects) of one organ without increasing the risk in at least one other organ. We design a system that can generate a large database of Pareto-optimal radiation treatment plans (30-100 plans) for an individual patient in a few hours. All these plans are good treatment plans according to the Pareto definition, but they emphasize different objectives of the treatment. We also develop and investigate a treatment plan navigator that allows the physician to browse through the candidate plans and find the most suitable plan for the individual patient interactively. In other words, our system will solve the mathematical side of the treatment planning problem by finding a set of Pareto-optimal treatment plans. We hypothesize that, based on this information, the physician can exercise clinical judgment in a more effective way.

Keywords: radiation therapy, treatment planning, Pareto optimality

158 Dynamic Multi-Organ Models to Improve Radiotherapy Treatment of the Liver

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Purpose/Objectives: The delivery of high-precision, conformal radiotherapy in the abdomen requires an accurate identification of the tumor and its boundaries, an understanding of the motion due to respiration, and accurate online guidance to deliver the radiation correctly at each treatment fraction. The susceptibility of the soft tissue in the abdomen to deformation makes the integration of multi-modality images for tumor definition and the matching of these planning images to the online images for guidance challenging. Deformable registration can improve the integration of image information and enable accurate tumor guidance. In addition, unresolved differences in geometry at the time of treatment delivery and difference in respiration motion, can be accounted for in the accumulated dose.

Materials/Methods: Patients previously treated on a free-breathing 6-fraction stereotactic liver radiotherapy protocol were analyzed. Contrast enhanced MR and CT images were obtained to identify the tumor boundaries. Cone-beam CT (CBCT) images acquired for daily image guidance were retrospectively sorted into 10 phases. The radiation dose was calculated based on exhale and inhale 4DCT images. Primary contours of the tumor and organs at risk were delineated on the exhale 4DCT and the liver contours were subsequently adapted to the inhale 4DCT and inhale/exhale CBCT datasets via model-based segmentation. A multi-organ biomechanical-model based deformable registration algorithm, MORFEUS, allowed tissue tracking across the breathing cycle and treatment fractions, and dose accumulation.

The differences in tumor defined on MR and CT and the improvements in concordance following deformable registration (over rigid registration) were computed. As the tumor is not visible on non-contrast enhanced CBCT images, rigid registration of the liver is traditionally used for image guidance. The improvements in tumor targeting using MORFEUS (compared to rigid registration of the liver) were calculated. The accumulated dose was calculated by tracking the patient throughout the treatment process. The static dose (D_{static}) is based on the exhale 4DCT from which the plan was generated, reflecting standard practice. The accumulated dose (D_{acc}) uses deformable registration to account for the daily variability in breathing motion and residual setup uncertainty, from each treatment 4D CBCT.

Results: Following deformable registration of the livers on planning CT and MR images, the population median of the average distance between the CT tumor surface and MR tumor surface was 3.7 mm (2.2- 21.3 mm). The average GTV concordance improved from 65% to 73% following deformable registration. Deformable registration improved tumor targeting, although the mean displacement of the COM following deformable registration was small, the maximum displacement of the COM was 0.34, 0.65, and 0.97 cm in the LR, AP, and SI directions, respectively. 15% of the treatment fractions had a COM displacement of greater than 0.3 cm and 33% of patients had at least 1 fraction with a displacement of greater than 0.3 cm. Changes in accumulated dose, relative to the planned (static) dose were seen: up to 7 Gy in mean dose to the liver, up to 4 Gy in maximum dose to critical normal tissues, and up to 1 Gy in minimum dose to the tumor.

Conclusions: Deformable registration with dynamic multi-organ models can improve the planning and execution of radiotherapy in the abdomen. In addition, it can improve the understanding of the accumulated dose, which can lead to a better understanding of dose -response/complication models.

Keywords: deformable registration, image guidance, radiation therapy

159 Tumor Microenvironment and Vasoresponse During Photodynamic Therapy as Determinants of Therapeutic Outcome

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Damage to tumor microvasculature has long been known to be an important mechanism of therapeutic action in photodynamic therapy (PDT) of solid malignancies. PDT, which involves the local illumination of disease that has accumulated an exogenously administered or endogenously produced photosensitizer, can lead to both increases and decreases in blood flow during illumination. In Photofrin-mediated PDT of murine radiation induced fibrosarcoma (RIF) tumors we have previously shown that within minutes of beginning illumination blood flow increases relative to pre-illumination levels, but then decreases at a rate that varies substantially among animals, even animals that were treated with identical PDT conditions. PDT effect on tumor blood flow in an individual animal is relevant to therapeutic outcome for that animal, as demonstrated by a highly significant association between the slope of decreasing blood flow during PDT and the time required for tumor regrowth to a volume of 400 mm^3 (Yu et al. Clin Cancer Res. 2005 11:3543-52). These data led us to hypothesis that therapeutic response to PDT could be augmented by approaches that alter vasoresponsiveness *during* illumination for PDT.

Vasoresponsiveness during PDT will be a function of the properties of the pre-existing vascular microenvironment so we have initiated studies to test our hypothesis by examining the effects of an altered tumor vascular microenvironment on the physiologic, mechanistic, and therapeutic effects of PDT. Comparative studies were performed between RIF tumors grown under standard conditions and those grown from cells co-injected with a small volume (15 μl) of Matrigel^{TM} in order to provide supplemental basement membrane for vascular development. Matrigel-supplemented tumors demonstrated a lower vascular density and were composed of vessels that were more evenly distributed, which was suggestive of a normalized vascular phenotype. Matrigel-supplemented tumors demonstrated less intratumor heterogeneity in vascular responses during PDT and, correspondingly, these tumors exhibited more prominent vascular shutdown after treatment. The potential advantage of a more homogeneous vascular response in Matrigel-supplemented tumors is reflected by a 36% cure rate after PDT of Matrigel-supplemented tumors versus a 0% cure rate after PDT of RIF tumors grown in standard conditions.

Keywords: photodynamic therapy, vasculature, normalization

160 Improving the Efficacy of Cancer Therapies by Defining and Exploiting Thermally Sensitive Vascular and Immunological Targets

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Vascular defects in the tumor microenvironment, together with related physiological consequences such as high interstitial fluid pressure, have been linked to inefficient and uneven intratumoral delivery of oxygen, therapeutic drugs and immune effector cells. Studies by others indicate that there are steep gradients of decreasing chemotherapeutic drugs concentration and increasing hypoxia surrounding perfused tumor blood vessels such that only the small fraction of tumor cells nearest to perfused blood vessels are ever exposed to drug. It seems clear therefore that improving the functional capacity of tumor blood vessels could broadly improve treatment efficacy. Our laboratory's research has been focused on learning more regarding the effects of mild, physiologically relevant, systemic hyperthermia on vascular and immunological targets. An overall hypothesis is that natural and long conserved vascular and immunological responses to elevated body temperature can be exploited to improve cancer therapies. This presentation will provide an update on two new and clinically relevant projects in our lab.

In one study, we are testing whether mild systemic hyperthermia can influence vascular perfusion and help reduce hypoxia in the tumor microenvironment. Hypoxia is a major barrier to effective radiation. In our studies, mice bearing tumors are given a mild, systemic thermal treatment (core temperature is brought to fever range, i.e., 39.5°C for several hours). Tumors (but not normal organs) from heat treated mice had significantly more perfused vessels than those from control mice as determined using an i.v. administered perfusion dye ($DiOC_7(3)$), a difference which persisted for several hours after body temperature returned to normal. Heat treatment also resulted in significant reduction in the hypoxic regions in tumors as estimated from imaging of Hypoxyprobe^{TM} stained tumor sections. This effect could be responsible for the fact that tumor growth in mice that received hyperthermia prior to radiation was significantly reduced in comparison to that seen in mice that received either treatment alone. In a second study, we are evaluating the role of fever range hyperthermia on neutrophil homeostasis and egress from bone marrow and the infiltration of neutrophils and other immune effector cells into the tumor microenvironment. In one case, we have observed that treating mice with mild (fever-range) whole body hyperthermia following a non-myeloablative dose of total body irradiation enhances neutrophil reconstitution through a thermally sensitive, cytokine driven bone marrow release of granulocytes to the peripheral blood. Other data from our group has characterized a thermally driven increase in the numbers of NK cells, neutrophils and macrophages into the tumor microenvironment.

Collectively, these observations suggest that manipulation of body temperature can selectively increase the number of perfused blood vessels in tumors and can help in the recruitment of important immune effector cells into the tumor microenvironment. Mild systemic heating could facilitate improved tumor control through several distinct mechanisms all of which support its application as an adjuvant for several cancer therapies. Research Supported by NCI grants: P01 CA94045 and R01 CA71599.

Keywords: hyperthermia, radiation therapy, tumor microenvironment

161 Surrogate Molecular Markers for Deposited Dose in Photodynamic Therapy With the New Photosensitizer HPPH

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Photodynamic therapy (PDT) is a two-step modality for the local treatment of early and advanced solid tumors. It entails the activation by light of a tumor-localized photosensitizer, which results in the formation of cytotoxic reactive oxygen species, predominantly singlet oxygen. Long-term cutaneous photosensitivity is an adverse effect of the FDA-approved photosensitizer porfimer sodium. The new photosensitizer HPPH (2-1[hexyloxyethyl]-2-devinylpyropheophorbide-a) may have at least equal efficacy as porfimer sodium without prolonged photosensitivity. We are currently embarked on a number of Phase I and II trials, including lung and H&N cancers, to establish the efficacy of HPPH.

Precise light dosimetry in areas of complicated geometry, such as the bronchoscopically accessible airways or the oral cavity, is a serious challenge in PDT. Therefore it is often difficult to determine whether regional treatment failure is due to failure to deliver an adequate light dose or to non-dosimetry related causes. One of our recent discoveries is the significant correlation between the PDT-induced generation of crosslinks of the intracellular STAT3 and PDT dose, i.e. biologic effectiveness of the PDT reaction. This was demonstrated in cells in vitro and preclinical tumor models. In a very limited number of samples from patients undergoing PDT for obstructive lung cancer we observed that absence of STAT3 crosslinks correlated with lack of treatment response. We have further observed that activation of the p38 stress MAPK mirrors the dose-dependent formation of crosslinked STAT complexes, but lacks the PDT-specific reporting function.

A Phase I/II clinical trial was initiated to test the efficacy of HPPH-PDT in the treatment of moderate to severe dysplasia, CIS and Stage I superficially invasive (<3mm thickness) squamous cell carcinoma, primary or recurrent, of the oral cavity, and to correlate molecular marker (STAT-3 crosslinks, p38) expression or lack thereof, as determined from biopsies obtained immediately following light exposure, with treatment response and overall patient outcomes. We believe that the light dose escalation design of the trial will provide the ideal circumstances for this correlation attempt. This information will significantly contribute to our understanding of the biological and dosimetry challenges of PDT.

Reference: Henderson BW et al., Clin. Cancer Res., 13: 3156, 2007.

Keywords: photodynamic therapy, photosensitizer, molecular markers

162 Optical Spectroscopy Informs Clinical Translation of Photodynamic Therapy

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Photodynamic therapy (PDT) is based on the optical excitation of a non-toxic photosensitizer. The absorption of light initiates a photophysical/photochemical cascade that, in the presence of molecular oxygen, creates highly reactive singlet oxygen, whose reactions with intracellular targets mediate a variety of biological responses that lead to local tumor control and cure. PDT has received limited regulatory approvals in the US and elsewhere, and clinical trials for a number of cancers are ongoing. Because PDT is inherently optical, it lends itself to optical methods of treatment planning and monitoring. Nearly all of the photosensitizers in clinical use fluoresce, and their fluorescence enables direct detection via imaging or spectroscopy. Many photosensitizers are degraded during PDT, and this degradation, known as photobleaching, may be useful in monitoring the progress of therapy. Reflectance spectroscopy can report tissue optical properties and hemoglobin oxygen saturation, both of which may change during therapy.

Preclinical work in our laboratory has resulted in robust means of acquiring photosensitizer fluorescence and reflectance spectra during PDT and means of analyzing these data. Recently, we have been successful in implementing these techniques in clinical trials of PDT for the treatment of superficial basal cell carcinoma and cutaneous T-cell lymphoma (CTCL) at Roswell Park Cancer Institute and Case Western Reserve University, respectively. In both cases, dedicated optical systems have been designed and built that enable integration of spectroscopic acquisition and approximately real-time display of results with the clinical treatment protocol. In the NCI-supported Roswell Park trial, we established for the first time in patients an irradiation fluence-rate-dependence of the rate of photobleaching of the sensitizer ALA-PpIX. Lower fluence rates were successful in eliminating pain during treatment, which has been a significant obstacle to more widespread clinical use of this form of PDT. Here also spectroscopy was useful in optimizing the delivery of PDT. Fluorescence photobleaching was monitored during the initial, low fluence rate phase of the protocol. When the sensitizer fluorescence had bleached to 10% of its pre-treatment level, the remainder of the prescribed light dose was delivered at a higher intensity, which resulted in a pain-free, time-efficient treatment[1]. The CTCL trial at Case Western is using a novel photosensitizer, Pc 4. Pc 4 has strong absorption at the treatment wavelength and is significantly more photostable than most sensitizers undergoing clinical evaluation. In a recently completed Phase I trial, our optical system found heterogeneity of Pc 4 levels among lesions and indicated that the presence of the Pc 4 significantly influences the optical properties of the skin and must therefore be accounted for in treatment planning[2]. Ongoing preclinical studies are directed at extending the spectroscopy methods to interstitial geometries required for treating deeper tumors.

References: [1] W.J. Cottrell et al. Clin. Cancer Res. In press 2008. [2] T.K. Lee et al., J. Biomed. Opt. 13:030507, 2008.

Keywords: skin, photodynamic therapy, spectroscopy

163 Individualization and Optimization of High Dose Conformal Radiation Therapy

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The overall objective of this work is the creation and study of improved radiotherapy treatments for brain, head/neck, lung and liver cancer made possible by new technologies, and their use for patient treatments which would not be possible otherwise. In this research, new developments in image-guided radiotherapy (IGRT), comprehensive adaptive radiotherapy optimization, quantitative MR and metabolic imaging are applied to our on-going clinical dose escalation or normal tissue complication probability (NTCP) reduction studies.

In this translational program project, new technical developments are used to make new clinical studies possible and practical. Research aimed at individualizing therapy includes development of a dose-to-date (DTD) infrastructure to test various scenarios of accumulating dose based on different assumptions about patient shape changes, from data acquired during the course of treatment, as well as optimizing threshold-based repositioning based on systematic and random setup error distributions and modifying the model of random variations from a population to the individual patient as treatment progresses. Various breath control strategies have been studied and clinically applied as part of lung and liver trials. The capability to map deformation between different imaging sets (and the dose associated with treatment corresponding to those image sets) is being used to study patient doses as the patient changes shape. Study of optimization capabilities needed for adaptive comprehensive planning includes creating fractionation-corrected bioeffect dose distributions and methods for simulating or approximating probability distributions for setup uncertainty and motion. Other work is studying incorporation of voxel-based 3-D functional imaging data (SPECT, PET) into optimization, so physical doses can be weighted spatially to spare more functional subunits (normal tissues) or to escalate preferentially a part of the target volume, and using Model Predictive Control to allow on-going monitoring of plan evaluation metrics to determine when adaptive replanning is necessary to maintain the quality of the overall treatment course.

New knowledge and capabilities developed within the project are applied directly as part of the clinical studies. Novel plan optimization techniques, improved patient setup and localization methods, and diffusion and perfusion MRI imaging have been applied to advanced Head/Neck treatments as part of a trial to reduce dysphagia by sparing the swallowing structures. This study has demonstrated the potential for intensity modulation (IMRT) to improve sparing of the swallowing structures and significant dose-effect relationships for all the swallowing structures. Concurrent study of dynamic contrast-enhanced MRI hypothesized that changes in tumor blood volumes early after the start of RT, compared with pre-RT, will be predictive of eventual response, and this DCE MRI data demonstrated significant differences in tumor blood volumes of patients who achieved CR compared with those who did not achieve CR at 3 months after RT. In an analogous set of clinical studies performed in glioblastoma patients, radiotherapy dose/fraction escalation using optimized Intensity Modulated Radiation Therapy (IMRT) plus concurrent temozolomide identified an association of 11C-Methionine PET uptake with site of failure.

Clinical studies of the lung and liver also use new technical capabilities to perform advanced radiation dose escalation trials. The current primary liver study is based on the novel capability to set the prescription dose limited by the iso-complication probability for normal liver damage while also evaluating the potential for use of liver perfusion (measured with dynamic contrast enhanced imaging) to assess RILD. An estimation method for liver perfusion was developed and applied, demonstrating that reductions in regional venous perfusion one month following RT were predicted by the local total dose and the change in the regional venous perfusion ($p<0.00001$). In addition, overall liver function showed significant correlation between indocyanine green clearance and the mean of the estimated portal vein perfusion in the functional liver ($p < 0.001$). Analagous dose escalation trials in unresectable lung cancer have made use of the iso-NTCP-based dose prescriptions for stage I/II and stage III protocols: for patients with GTV >51.8 cm3, the median overall survival in those with BED (biological effective dose) >79.2 Gy and < 79.2 Gy were 30.4 and 18.2 months, respectively ($p < 0.001$), while there was no significant difference for smaller GTVs. These trials also include pilot studies of feasibility of

using PET and SPECT to assess tumor response and normal lung injury, as well as correlation of radiation induced lung toxicity with elevation of plasma TGFbeta levels.

Integration of the technical development efforts and clinical research studies within the same program project have led to the ability to quickly and effectively feed back needs from the clinical studies to the technical research efforts, and for new capabilities to be incorporated into the design of clinical studies. This integrated translational research program has made possible the development and clinical testing of conformal radiotherapy, computer-controlled treatment delivery, dose escalation, normal tissue complication probability-driven protocols, IMRT, image guided radiation therapy (IGRT) and now metabolic and functional imaging capabilities.

Keywords: radiotherapy dose escalation, IMRT and IGRT optimization, imaging for treatment response prediction

164 Photodynamic Therapy for Serosal Malignancies: An Integrated Bedside to Bench Approach To Translational Research

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The treatment of serosal surface malignancies, including recurrent peritoneal carcinomatosis resulting from ovarian and gastrointestinal cancers and pleural carcinomatosis commonly resulting from non-small cell lung cancer (NSCLC), is typically palliative in nature. We have investigated the use of surgical debulking followed by intraoperative photodynamic therapy (PDT) in the treatment of serosal malignancies.

PDT combines photosensitizer, light and oxygen to mediate cancer cell cytotoxicity through a combination of direct effects on cancer cells and indirect effects on tumor microenvironment. We have developed novel instrumentation that allows real-time measurement of these three key elements of PDT and applied this technology to pre-clinical models and clinical trials of PDT. Using these techniques to deliver PDT with increased accuracy and homogeneity, we have found PDT can be an effective treatment for patients with serosal malignancies. In a phase II trial of surgery with pleural PDT for patients with pleural spread of NSCLC, we have attained a 22 month median survival and 73% local control at 6 months. A phase II trial of surgery with peritoneal PDT for patients with recurrent ovarian and gastrointestinal peritoneal carcinomatosis showed median survivals of 20 and 11 months, respectively. Nevertheless, these studies showed that the therapeutic index of serosal PDT is significantly limited by the intrinsic heterogeneities in tumor photosensitizer uptake, optical properties and oxygenation/blood flow.

Therefore, we have taken an integrated approach to improving the safety and efficacy of serosal PDT. We are continuing to develop technological innovations that will allow us to make spatially resolved, real-time measurements of the relevant optical and physiologic parameters in both tumor and normal tissues and to deliver light in ways that compensate for tumor heterogeneity. We have also approached this problem biologically by using modifiers of cellular signal transduction to promote an anti-tumor tissue microenvironment and to inhibit the pathways that allow cancer cells to survive PDT-mediated damage. Finally and most importantly, we have integrated the findings from these preclinical studies into our ongoing clinical trials of serosal PDT to both validate and refine our preclinical models and to gain critical insights into novel approaches to enhancing the therapeutic index of serosal PDT.

Keywords: photodynamic therapy, lung cancer, ovarian cancer

165 Radiation-Induced TNF-alpha Therapy in Prostate Cancer

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Radiotherapy (RT), in combination with androgen ablation is a standard treatment for patients with poor prognosis localized prostate cancer. Nevertheless, outcomes are suboptimal in part due to inadequate local control, which may result in eventual spread to distant sites. The addition of radiosensitizing agents would potentially increase local control at current radiation doses, or potentially allow patients to receive lower RT doses with associated lower toxicities. TNF-α is a potent radiosensitizing anti-tumor agent, but toxicity limits its use as a systemic drug. Ad.Egr-TNF.11D (TNFerade™, GenVec, Gaithersburg, MD) is a replication deficient E1, E3, E4 deleted adenoviral vector that encodes RT-inducible sequences upstream from a cDNA for human TNF-α. In pre-clinical models, RT-induced intratumoral TNF-α results in enhanced tumor regression via vascular destruction and thrombosis. Phase I and II trials of Ad.Egr-TNF.11D and RT have demonstrated safety and potential efficacy in sarcoma, esophageal, head and neck and rectal cancers, and is currently undergoing phase III evaluation in pancreatic cancer. Patients with high risk localized prostate cancer have an increased incidence of local recurrence, which may translate into distant recurrence and decreased survival making it a viable clinical venue for further clinical and translational research. Indeed, higher radiotherapy doses have led to improved disease control, but further improvements are limited by current technology, cost and normal tissue toxicity.

Based on these data, adding Ad.Egr-TNF.11D to standard radiotherapy and androgen ablation has potential, but requires formal safety evaluation. A phase I study incorporating a novel Bayesian continuous toxicity monitoring scheme (TITE-CRM) has thus been planned. Even as clinical development of Ad.Egr-TNF.11D with RT proceeds, the mechanisms mediating resistance and treatments to overcome such resistance need to be determined. Furthermore, selection of patients and tumors resistant to standard RT would help define the population for future phase II and III trials. Our data to date suggest that RT not only induces STAT1, but that STAT1 and an associated 7-gene expression profile are mechanistically important for radiation resistance, raising the hypothesis that these may be predictive biomarkers. In addition, activation of NFκB by both TNF and radiation may be critical to promoting survival and inhibiting both the cancer and endothelial cell death required for successful treatment. Further preclinical studies inhibiting NFκB activation with small molecules in the context of this treatment are in process. Ongoing studies utilizing SPORE tissue resources furthermore seek to determine the prognostic and predictive value of STAT1 and the associated 7-gene signature in patients previously treated with surgery or RT.

Keywords: gene therapy, radiotherapy, TNF-alpha

166 Plasminogen Kringle 5 Induces Apoptosis of Brain Microvessel Endothelial Cells: Sensitization by Radiation and Requirement for GRP78 and LRP1

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Recombinant plasminogen kringle 5 (rK5) has been shown to induce apoptosis of dermal microvessel endothelial cells (MvEC) (Davidson et al., 2005). As we are interested in anti-angiogenic therapy for glioblastoma tumors and the effectiveness of anti-angiogenic therapy can be enhanced when combined with radiation, we investigated the pro-apoptotic effect of rK5 on brain MvEC plus/minus prior irradiation. We found that rK5 treatment of brain MvEC induced apoptosis in a dose- and time-dependent manner, measured as the cleavage of caspase-7 or 3 and TUNEL positivity. Prior irradiation significantly sensitized (500-fold) the cells to the pro-apoptotic effect of rK5. The pro-apoptotic effect of rK5 required expression of glucose regulated protein 78 (GRP78), based on blocking studies with an antibody directed toward GRP78 and the downregulation of GRP78 with small interfering (si) RNA. In addition to GRP78, the pro-apoptotic effect of rK5 post-irradiation required the low density lipoprotein receptor-related protein 1 (LRP1), a scavenger receptor. The necessity for LRP1 was demonstrated by blocking the pro-apoptotic effect of rK5 with a competitive inhibitor of ligand binding to LRP1, recombinant receptor-associated protein, and by the downregulation of LRP1 with siRNA. Our findings have potential application as a new therapy for glioblastoma tumors, as we also demonstrate that the expression of GRP78 protein is upregulated on brain MvEC in glioblastoma tumor samples as compared to the normal brain. Immunoblotting studies confirmed the upregulation of GRP78 in the tumor samples. Overall, these data suggest that irradiation sensitizes brain MvEC to the pro-apoptotic effect of rK5 and that this signal requires LRP1 internalization of GRP78. In summary, these studies suggest rK5 maybe an important new anti-angiogenic therapy, particularly when combined with radiation, for malignant gliomas and likely other cancers.

Keywords: plasminogen kringle 5, glioblastoma, angiogenesis

167 *In Vivo* Imaging of Tumor Oxygen Levels and Glycolytic Activity Using Paramagnetic Resonance Imaging

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Aerobic glycolysis may be an adaptive response to hypoxic conditions [chronic and intermittent] in tumor by up-regulating glucose uptake and metabolism. Tumor hypoxia confers resistance to radiotherapy. *A priori* knowledge of spatial and temporal changes in partial pressure of oxygen (oxygenation; pO_2) in solid tumors, a key prognostic factor in cancer treatment outcome, could greatly improve treatment planning in radiotherapy and chemotherapy. A low field MRI approach, called Electron Paramagnetic Resonance Imaging (EPRI) using an oxygen sensing paramagnetic contrast media, is described which for the first time provides non-invasive three-dimensional maps of tumor pO_2. We developed a unified EPRI and MRI system that enabled generation of pO_2 maps with anatomic guidance. The system demonstrated a sensitivity to distinguish differences in pO_2 of ±3 mmHg, enabling it to recognize radiobiologically hypoxic regions in tumors on an absolute pO_2 basis, a capability unique to EPR imaging. Further, with information such as blood flow, blood volume available from MRI and metabolite levels from MRS, it was possible to examine the relationship between flow related issues, metabolic status for the first time and tissue pO_2 obtained by EPRI. Oxygen images from EPR imaging studies show that tumor oxygen maps displayed heterogeneity with both hypoxic and relatively well oxygenated regions. MRS spectra obtained from these regions show that a strong lactate peak was observed in even well oxygenated regions, suggesting the predominance of aerobic glycolysis processes in this tumor. Total choline peak also positively correlated with tumor pO_2.

This technique, combining functional and anatomic imaging, shows preclinical applicability in monitoring factors that control tumor hypoxia and metabolism and may have future clinical potential for monitoring tumor response to treatment

Keywords: tumor, Imaging, metabolic

168 A Phase I Trial and Molecular Tissue Analysis of Capecitabine Concurrent with Radiation Therapy for Patients Newly Diagnosed With Glioblastoma Multiforme

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Glioblastoma multiforme (GBM) tumors are the most frequent and most lethal primary intracranial malignancies of the central nervous system with a median survival time of 12-15 months. Pharmacogenomic studies have correlated the antitumor response to Capecitabine (a novel, fluoropyrimidine prodrug) with the expression of the drug metabolizing enzymes thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD). We examined TP and DPD expression in normal human brain tissues and in GBM. Because previous reports suggested an increase in TP expression after irradiation (a current standard of care for malignant gliomas), we also examined the effect of irradiation on the expression of TP and DPD, both irradiated and lead-shielded contralateral U87MG glioma xenografts within the same animal. Expression levels, determined using real-time quantitative PCR, demonstrated an approximately 70-fold increase in TP mRNA levels 4 days after irradiation, relative to initial control levels. Simultaneously, there was no increase in DPD, an enzyme that catabolizes fluoropyrimidines to non-toxic β-alanine. Interestingly, TP mRNA in the lead-shielded tumors (contralateral to irradiated tumors) increased approximately 60-fold by day 10 relative to initial control levels, suggesting an abscopal effect. Elevated TP levels were sustained for 20 days in irradiated xenografts. These published results (Blanquicett et al., 2002) formed the rational basis for a Phase I trial to examine the maximum tolerated dose (MTD) of capecitabine (CAPE) when administered concurrently with radiation therapy (RT) in patients with newly diagnosed GBM. Secondary endpoints include safety, time-to-progression (TTP), overall survival (OS), and gene expression analysis of resected GBM specimens. Patients were enrolled in 2 cohorts based on administration of P450-inducing or non-inducing anti-epileptic drugs. During induction, patients received CAPE starting at 625 mg/m^2 BID (1250 mg/m^2/day) and escalated by 25% in 3 patients per cohort on a 6 week continuous basis concurrent with RT (60 Gy in 30 fractions) followed by 4 weeks CAPE only. After a 1-week hiatus, patients received maintenance CAPE at 1,250 mg/m^2 BID (2,500 mg/m^2/day), on days 1-14 every 3 weeks until progression or unacceptable toxicity. Resected GBM specimens were obtained prior to initiation of chemoradiation therapy. The expression of 94 genes involved in CAPE metabolism and/or response to RT were assessed using the Taqman low-density-array (TLDA). From December 2002 to April 2004, 18 patients were enrolled. The MTD for both arms was 625mg/m^2 BID. Median TTP and OS were 248 and 366.5 days respectively. Statistically significant correlations were identified between expression of 28 genes and TTP and OS. Concurrent CAPE and RT for newly diagnosed GBM appears safe and well-tolerated without unexpected toxicities. This pharmacogenomically-designed treatment modality includes genetic analysis of tumor tissues correlating clinical outcome (TTP and OS) to potential biomarkers for stratification in future clinical trials.

Keywords: Capecitabine (Xeloda®), glioblastoma, chemoradiation

169 Understanding Underlying Mechanisms and Optimizing Photodynamic Therapy (PDT)

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Photodynamic therapy (PDT) is a three component process involving a non-toxic, tumor-avid photosensitizer (PS), activating light and molecular oxygen producing singlet oxygen and other oxidative species that cause widespread cellular damage, alterations in signaling pathways and *in vivo* host responses. PDT induces cell death by apoptotic, necrotic and autophagic mechanisms. It also can shut down tumor vasculature, and induce host inflammatory and systemic immune responses. PDT is effective clinically, but the complex dosimetry and underlying molecular and tissue-level mechanisms are not well understood, nor has there been substantial investigation of rational combinations of PDT with agents targeting intracellular signaling pathways.

We have been designing and evaluating new PS including HPPH (2-1[hexyloxyethyl] -2-devinyl pyropheophorbide-a), a second generation agent optimized by *in vivo* structure-activity studies in preclinical models that now is in PII clinical trials for H&N, esophagus, and lung cancers. We also are developing multimodality agents combining fluorescence and/or PET-based imaging with tumor targeted PDT[1].

We found the ABCG2 transporter removes many clinical PS from tumor cells including cancer stem cells, but that transport is inhibited by TKIs such as imatanib mesylate (Gleevec); inhibition at the time of PS administration enhances *in vivo* PDT[2]. We plan PI/II clinical trials of effects of Gleevec with PDT. We also find that combining PDT with inhibitors of the cMET receptor and mTOR pathway enhances preclinical efficacy in cells and *in vivo*; this work will be extended to PI/II clinical trials. As discussed in the abstract by TH Foster, PS auto-oxidization (photobleaching) kinetics are a metric for efficiency[3]; and we plan PII clinical trials of topical ALA-PDT for superficial and nodular basal cell carcinoma (BCC) and in situ squamous cell carcinoma to utilize PS photobleaching rates to establish optimum irradiances and minimal pain, and then to extend the trials to determine the dose-response relationships and recurrence rates at these low irradiances.

The oxidative reactions in PDT and the multiple cell death pathways generate new epitopes and tumor associated antigens. In addition, the inflammatory reactions create a milieu that matures dendritic antigen presenting cells. Our investigation into the ability of PDT to enhance anti-tumor immunity led to the novel discovery that PDT-treated tumor cells are effective anti-tumor vaccines[4]. A pilot clinical study showed for the first time that PDT of BCC augments patient reactivity to a tumor associated antigen[4]. In preclinical models we found that ex vivo, PDT- generated vaccines can be used adjuvantly to induce systemic anti-tumor immunity. We are extending this finding to PI trials of autologous, PDT-generated *ex vivo* vaccines against melanomas, and also against BCCs in patients with nevoid basal cell carcinoma syndrome who develop 10's- 100's of carcinomas per yr.

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Keywords: photodynamic therapy (PDT), ABCG2, anti-tumor immune responses

170 Realistic Phantoms for Clinical and Preclinical Dose Calculations

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We have developed a series of anatomically realistic phantoms representing adults and children, as well as realistic models of mice (20, 25, and 30 g body weight) and rats (200, 300, and 400 g), using body models based on non-uniform rational B-splines (NURBS). The human organ and body masses were based on the reference mass values for male and female adults, newborns, 1-, 5-, 10- and 15-year-olds given in ICRP Publication 89. These models were used to develop dose factors for use in internal or external dose calculations, in nuclear medicine and other applications. The body and organ models were scaled and shaped using a software tool developed in Visual C++. Voxelized versions of these models were used in the GEANT4 radiation transport code for calculation of specific absorbed fractions (SAFs) for internal radiation sources. We scaled the human models to within a few % of ICRP-89 reference masses, with the models reviewed by physicians for anatomical realism. For the animal models, existing MOBY and ROBY models were scaled to develop animals of different size, as are used in preclinical investigations. Development of individual phantoms was much faster than via manual segmentation of medical images, and resulted in a very uniform standardized phantom series. SAFs for discrete photon and electron sources were developed, using standard starting energy values, using a multinode computing network (ACCRE). Photon and electron SAFs were calculated for all organs in all models, and were compared to values from similar phantoms developed by others. Agreement was very good in most cases; some differences were seen, due to differences in organ mass and geometry. This realistic human phantom series represents an update on the Cristy/Eckerman phantom series of the 1980's; the animal model series is the first of its kind. All phantom sets will be included in the next release of the OLINDA/EXM personal computer software for internal dose assessment, and the phantoms will be made available for general use by the biomedical community.

Keywords: radiation dosimetry, models, Monte Carlo

171 Update on Survival From the Original Phase II Trial of Bevacuzimab and Irinotecan in Recurrent Malignant Gliomas

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Background: Recurrent grade III-IV malignant gliomas have a dismal prognosis and effective salvage therapies are limited.

Methods: From 4/05-2/06, a phase II trial was conducted at Duke University using bevacuzimab and irinotecan in patients with recurrent malignant gliomas. 2 cohorts were enrolled that included 33 grade III and 35 grade IV patients. The first cohort received bevacuzimab at 10mg/kg plus irinotecan (dose based on patient's anticonvulsant) every two weeks. The second cohort received bevacuzimab at 15mg/kg every 21 days and irinotecan on days 1, 8, 22, and 29.

Results: Overall response rates for both grade III and IV were 59% (grade III 61%, grade IV 57%). 6 month PFS and OS for grade III were 59% and 79% and for grade IV 43% and 74% respectively. In 12/07, we evaluated all patients enrolled in the trial to determine the 2 yr OS. From the 2 cohorts, 22% (15/68) of the patients are still alive (11 grade III, 4 grade IV). For the grade IV patients, the 2yr OS is 15%. All four of the grade IV patients completed 9 cycles of therapy. Two (2/4) progressed (8mo and 17mo) and both reinitiated bevacuzimab and irinotecan with radiographic response. The other two have been progression free since the end of treatment (11mo and 18mo). Surprisingly, both of these patients had only partial resections at the time of diagnosis. For the grade III patients, the 2 yr OS is 33%. All but one patient has progressed; ranging from 1 to 14 months. 4 patients are currently on bevacuzimab-based therapy. 1 on carboplatin, 2 on etoposide and 1 on bevacuzimab alone for radiation necrosis. The remaining patients are on metronomic temozolomide (2), etoposide (1), and a phase I clinical trial (2). 2 patients are currently being followed off therapy. The one patient who did not progress only received a partial cycle on study and had to discontinue secondary to TTP and has been off treatment for the last 21 months.

Conclusion: The combination of bevacuzimab and irinotecan provides a clinically meaningful treatment option for patients with recurrent malignant gliomas.

Keywords: malignant glioma, bevacizumab, irinotecan

172 Uncertainty-Informed Image-Guided Adaptive Radiation Therapy: Towards Optimal Decision Making From Imperfect Data

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The major goal of our research program is to improve radiation therapy outcomes by more accurately targeting high dose delivery to tissues with high tumor burdens, avoidance of organs at risk for complications. Our approach, image-guided adaptive radiation therapy (IGART), applies deformable image registration (DIR) to daily onboard kilovoltage x-ray cone-beam computed tomography (CBCT) images to create four-dimensional (4D) representations of patient anatomy (4D voxel trajectories). 4D voxel trajectories describe setup error, organ position and shape, and target volume changes as functions of time, providing the basis for adapting treatment on a weekly, daily, or even real-time basis. Another task is integrating IGART with non-anatomic PET- and MR- based imaging modalities which have the potential to more accurately identify tissues at risk. IGART seeks to reduce systematic and random tissue localization errors (2-5 mm for conventional RT) enabling significant reduction of the 8-15 mm planning target volume (PTV) margins currently needed to ensure target volume coverage.

A major challenge is accommodating the relatively high level of uncertainty associated with the underlying IGART registration and imaging modalities. For example, available DIR validation studies suggest that estimated displacement vector fields (DVFs) have typical errors of 1-4 mm. Published observer segmentation studies show prostate boundary delineation errors range from 1 to 4 mm for CT and MR imaging. Recently reported sensitivities and specificities for MR spectroscopic delineation of intraprostatic tumor are 46-78% and 86-93%, respectively. Optimal selection of biological IGART plans must take into account the uncertainty and incompleteness of available 4D anatomic representations.

To address this problem, we are developing methods for quantifying uncertainty of an estimated DVF, DVF_{est}, in terms of the probability density function (pdf), $p(DVF_{est} - DVF_{true})$, where DVF_{true} is the actual deformation. By incorporating this pdf into the IGART optimization process, an adapted plan can be selected that has a high probability of satisfying treatment goals and constraints. To demonstrate this process, the probable distribution of registration errors due to ignoring breathing motion will be extracted from a set of 13 4D CT scans of lung cancer patients in which each breathing phase has been deformably registered to the peak inhalation phase (*Med. Phys. 35: 1251*). The method consists of expanding DVF in terms of nonorthogonal B-spline basis functions followed by applying principle components analysis to the resulting set of expansion coefficients.

Supported by NIH Grant P01 CA 116602.

Keywords: radiation therapy, image guidance, plan optimization

173 An Image-Guided Small Animal Radiation Platform for Preclinical Research

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There are at present significant technological disparities between the irradiation methods employed for clinical radiation therapy and laboratory radiation research. In the clinic, advanced three-dimensional (3D) and computer-controlled delivery technologies are now used to deliver fractionated conformal and intensity modulated radiation treatment. In the laboratory, however, simple single beam/single fraction arrangements are commonly used for animal irradiation. The significant differences in the dosimetry delivered to the tumors and the surrounding organs are major hurdles in the pre-clinical assessment of novel radiation treatment methods, alone or in combination with other therapeutic agents.

The Small Animal Radiation Research Platform (SARRP) is constructed to mimic a modern radiation treatment machine with integrated imaging, radiation delivery and treatment planning capabilities. The SARRP spans 3 ft x 4 ft x 6 ft (WxLxH). A constant voltage x-ray source with dual-focal (0.4 mm and 3.0 mm) spots is mounted on isocentric gantry. The source to isocenter distance is 35 cm, large enough to accommodate rodents ranging from mice to rabbits. Robotic translate/rotate stages are used to position the animal. The smaller focal spot is used for imaging with 80-100 kVp x-rays. For image guided irradiation, a pre-irradiation cone-beam CT (CBCT) is acquired by rotating the horizontal animal between the stationary, and oppositely mounted, x-ray source and flat panel amorphous silicon detector. Scans with voxel resolution of (0.55 x 0.55 x 0.55) mm^3 are acquired with less than 1 cGy in 4 min. Both focal spots are employed for irradiation experiments and operate at 225 kVp with 0.5mm added copper filtration. Radiation beams can be collimated from 0.5 mm in diameter to (60 X 60) mm^2. Treatment planning based on Monte Carlo dose calculations is performed and visualized at sub-mm resolution. Conformal dose distributions are delivered using a combination of gantry and robotic stage motion. The isocenter dose outputs at 1 cm depth in water are high; ranging from 100-375 cGy/min for the smallest to the largest radiation fields, respectively. The 20% to 80% dose fall-off is steep, spanning 0.16 mm. With CBCT guidance, the SARRP is capable of delivering 3D conformal radiation with an accuracy of less than 0.5 mm, as demonstrated by exposing film embedded in a phantom. This focal irradiation capability is also verified in vivo by means of γ-H2AX phosphorylation staining of a planned irradiation region of a mouse brain.

The ability of our system to focally irradiate a specific anatomic region or target in a laboratory animal has generated exciting new collaborations in pre-clinical research. These include the study of the response of normal tissue and tumor to focal radiation injuries in the brain; the development of molecular imaging markers for early assessment of radiation induced toxicity in the lungs; and the study of molecularly targeted therapy in combination with radiation for prostate and pancreatic cancers. We are hopeful that our SARRP, in combination with other molecular imaging modalities, will provide a timely and powerful technology to greatly transform future cancer treatment.

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Keywords: image guided focal irradiation, small animal radiation research, pre-clinical investigations

174 Metabolic Syndrome Following Transplant for Leukemia

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Adult survivors of acute leukemia in childhood have a higher than expected frequency of obesity early mortality from cardiovascular disease, and an increased risk for the *metabolic syndrome*, a constellation of disorders characterized by central obesity, insulin resistance, glucose intolerance, dyslipidemia, and hypertension. These long term adverse effects may be even higher in individuals who received hematopoietic cell transplant (HCT) as part of their therapy. The major objective of this ongoing research is to evaluate the relationship between transplant specific treatment exposures and the development of insulin resistance and the metabolic syndrome in adolescent and young adult survivors who underwent HCT for treatment of childhood hematologic malignancies. Studies in HCT survivors with metabolic syndrome and/or insulin resistance are being compared to studies in insulin sensitive survivors and with normal age/sex matched control subjects to determine the effects radiation (total body (TBI) and cranial radiation), drug therapies (glucocorticoids, high dose chemotherapy) and post-HCT behavioral changes (diet, physical activity) on development of the metabolic syndrome and individual cardiovascular risk factors. The aims of this study are (1) to obtain measurements of insulin resistance (euglycemic hyperinsulinemic clamp) in 190 survivors of HCT for hematologic malignancy and 190 age/sex frequency matched controls and to obtain other measurements of factors associated with obesity and metabolic syndrome. (2)To determine the association of the HCT preparative regimen (TBI, chemotherapy) and post-transplant treatments (glucocorticoids) with the development of the metabolic syndrome in HCT survivors, (3) to measure inflammatory factors [interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP)] and adipokines (adiponectin, leptin) in HCT survivors and controls, (4) to evaluate the association of metabolic syndrome and treatment exposures on the development of early signs of impaired endothelial function and cardiovascular changes in HCT survivors and controls and (5) to obtain measures of dietary intake and physical activity in HCT survivors and controls. Recognition of the transplant related risk factors that are significant for the development of metabolic syndrome early in the post-HCT time period, before overt disease develops, will provide information required to design intervention strategies with the aim of prevention and/or intervention and additional study of at risk individuals.

Keywords: metabolic syndrome, stem cell transplantation, cardiovascular disease

175 Metabolic Syndrome Following Transplant for Leukemia in Childhood Cancer Survivors

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The MS, composed of central obesity, dyslipidemia, hypertension, and insulin resistance (IR) is a clustering of potent risk factors for premature cardiovascular disease in adults and type 2 diabetes. The syndrome is also present in children and its prevalence in otherwise healthy children and adolescents was 6.8% in the most recent available data from NHANES 1999-2000. In healthy populations obesity has a central role in the development of the syndrome. A higher than expected frequency of obesity and early mortality from cardiovascular disease has been reported in adult survivors of childhood cancer. Although limited, there is evidence to indicate that increased risk for the MS is already present in childhood cancer survivors (CCS) early in life. Recent evidence from pilot data obtained at the University of Minnesota has shown a similar prevalence (16%) of MS in adult survivors of ALL. In a group of children who survived cancer for a median of 8 years, CCS had substantially higher risk of insulin resistance, hypertension and lipid abnormalities, despite a prevalence of obesity similar to the general population. Little is known about the pathogenesis of the syndrome in CCS, and it is conceivable that additional mechanisms other than obesity may play a critical role in the development of metabolic syndrome in this population. A number of antineoplastic agents, as well as hormonal deficiencies, in particular growth hormone deficiency, which can result from damage to the hypothalamic-pituitary axis from either chemotherapy or cranial radiation, have been associated with the metabolic syndrome in cancer survivors. This ongoing clinical study is evaluating the relation between IR, growth hormone deficiency, other mediators of IR and specific treatment regimens used to treat cancer, with the development of MS in a sample of children and adolescents who survived childhood cancer and to compare the prevalence of MS, IR, and lifestyle factors in CCS with a control population represented by healthy siblings, frequency matched by age and gender, who are being studied in a similar fashion. Also being investigated is whether endothelial impairment and other early signs of cardiovascular disease are significantly more prevalent among the CCS with MS, compared to those without MS and whether they are associated with specific therapeutic regimens. This study will provide compelling data that is not present in the current literature on the risk of the MS in CCS . Moreover, studying the components of the MS early in life, will provide insight into their sequence of development before the interactions become complex and disease develops. This information may also provide the opportunity to design future studies aimed towards prevention/ intervention for at risk individuals.

Keywords: metabolic syndrome, stem cell transplantation, cardiovascular disease

176 Growth Inhibitory Effect of a Low-Fat Diet on Prostate Cancer Cells *in Vitro*: Results of a Prospective Randomized Dietary Intervention Trial in Men With Prostate Cancer

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Purpose: A high-fat Western diet and sedentary lifestyle may predispose men to develop prostate cancer through changes in serum hormones and growth factors. In a prior retrospective human intervention study, we demonstrated that a low-fat diet combined with exercise reduced serum-stimulated growth of LNCaP cells in an ex vivo bioassay. We sought to further evaluate the effect of a low-fat diet on serum factors impacting on prostate cancer growth by conducting a prospective randomized dietary intervention trial in men with prostate cancer.

Materials A\and Methods: 18 men with prostate cancer that had not received prior therapy were randomized to a low-fat (15% Kcal), high fiber, soy protein supplemented diet vs. a Western (40% Kcal fat) diet for 4-weeks. Fasting serum was collected at baseline and following the intervention for measurement of sex hormones, lipids, fatty acids, IGF-I, IGF-II, IGF binding proteins, and PSA. LNCaP cells were cultured in media containing pre and post-intervention human serum to assess the effect of the diet on prostate cancer growth. Compliance was insured by having all meals prepared at the UCLA Clinical Research Center and close monitoring by a research dietitian.

Results: Subjects in both groups were highly compliant with the dietary intervention. Serum from men in the low-fat group significantly reduced in vitro growth of LNCaP cells relative to Western diet serum ($p=0.006$). Serum triglyceride and linoleic acid levels (omega-6 polyunsaturated fatty acid, the predominant fatty acid in the Western diet in baked foods) were significantly reduced in the low-fat diet group ($p=0.034$ and 0.005 respectively). Correlation analysis demonstrated that reduced omega-6 fatty acid levels and increased omega-3 fatty acid levels correlated with decreased serum-stimulated growth of LNCaP cells ($r=0.64$, $p=0.004$; $r=-0.49$, $p=0.04$).

Conclusion: In this prospective randomized dietary intervention trial, a low-fat diet resulted in changes in serum fatty acid levels that were associated with decreased growth of human LNCaP cancer cells in an ex vivo bioassay. Further studies are indicated evaluating if ex-vivo bioassay results correlate with clinically significant endpoints in prostate cancer patients. Further prospective trials are also indicated evaluating low-fat diets and omega-3 fatty acids for prostate cancer prevention and treatment.

Keywords: prostate cancer, dietary intervention, growth inhibitory effect

177 Puberty and Polyphenol-Containing Diets and the Risk of Breast Cancer

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The Center for Nutrient-Gene Interaction (CNGI) has examined the hypothesis that exposure to polyphenols (genistein, GEN; resveratrol, RES; and epigallocatechin-gallate, EGCG) in the diet at the time of puberty alters the programming of the development of the mammary gland and in doing so reduces cancer risk in adult life. Three major themes have been explored. The main hypothesis has been examined experimentally in a rat model of breast cancer; an observational cohort study has been conducted in girls going through puberty to determine if a soy diet influences the onset of menarche (a risk factor for breast cancer) or other markers of pubertal development; finally, the statistical issues associated with experimental design and the validity of post experiment data analysis in high dimensional biology have been studied from a theoretical standpoint. These themes were supported by core facilities specializing in genomic and microarray analysis, proteomics analysis, mass-spectrometry, and biostatistics/bioinformatics. A pilot project program and a career development program completed the activities of CNGI. Animal studies revealed that GEN and RES, but not EGCG, were chemopreventive when given in the diet during the period up to weaning (0-21 days). This preventive effect was associated with increased apoptosis in the mammary gland at the time of administration of the carcinogen (50 days). The 2-year cohort study (230 girls consuming low (<4 mg/d) or high (>12 mg/d) amounts of isoflavones) has just concluded and statistical analyses are underway. Urinary isoflavone analysis will be used to verify soy intake data. Urines collected in this study are being used for a new NCCAM-funded R21 grant to identify peptides that are associated with the stages of menarche. Statistical research led to the generation of methods to estimate the power of a proposed microarray analysis (HDBStat!) and to identify damaged microarray chips (the Geography Index). Microarray experiments on mammary glands from the rat experiment revealed several changes in metabolism, particularly a fall in expression of all Krebs cycle genes, as the mammary gland progresses from weaning (day 21) to time of administration of the carcinogen (day 50). Quantitative RT-PCR and Western blot experiments confirmed the fall in the Krebs cycle gene expression and protein abundance; GEN and RES restored the loss. 2D-gel and Gel-LC-tandem mass spectrometry identified 474 proteins associated with the mammary gland, 65 of which are changed between day 21 and day 50. Overall, the data suggest that the engines of the cell, the mitochondria, fall at a critical time with respect to the greatest sensitivity to carcinogens and that this fall is prevented by dietary polyphenols. The outcome of this study reinforces the concept that diet and exercise in adolescents is critical in lowering breast cancer risk.

Keywords: adolescence, mitochondria, breast cancer

178 Behavioral Modulation of Genetic Predisposition to Hepatocellular Cancer

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Although Hepatocellular Carcinoma (HCC) has long been etiologically associated with viral hepatitis, alcohol and other toxins, an increasing number of HCC cases now appears to be related to the pandemic of obesity and its consequent comorbidities of fatty liver and non alcoholic steatohepatitis (NASH). Our studies of diet-induced obesity susceptible (C57BL/6J) and resistant (A/J) inbred strains of mice, demonstrate both a behavioral and genetic component to the development of obesity related HCC. The behavioral component should be amenable to lifestyle interventions whereas the genetic component provides an opportunity to define risk markers to identify candidates most likely to benefit from a behavioral lifestyle intervention.

C57BL/6J but not A/J males fed a High Fat/High Sucrose (HF/HS) diet developed characteristics of the metabolic syndrome including obesity, insulin resistance, hyperglycemia and dyslipidemia. Neither strain developed these conditions when maintained on a Low Fat/Low Sucrose (LF/LS) diet. C57BL/6J male mice maintained on the HF/HS diet also developed hepatic steatosis, hepatitis, fibrosis, dysplasia and HCC. Neither A/J mice, fed the same HF/HS diet, nor C57Bl/6J or A/J mice fed the LF/LS diet showed hepatic steatosis, NASH or HCC. Interestingly, behavioral modification, consisting of diet switch from HF/HS to LF/LS at 135 days of age, reversed development of obesity and protected against development of HCC in C57BL/6J mice.

In summary, these studies demonstrate a unique model for behavioral-genetic interaction contributing to or protecting against HCC. A lifestyle program of a HF/HS diet leads to obesity, metabolic syndrome and HCC in a genetically predisposed mouse strain. In contrast, a lifestyle program of eating a LF/LS diet prevents development of all these comorbid conditions. The behavioral modification of switching diets from HF/HS to LF/LS reverses obesity and protects against development of HCC.

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Keywords: hepatocellular cancer, behavioral modification, genetic predisposition

179 Translational Chemoprevention Studies With Chlorophylls

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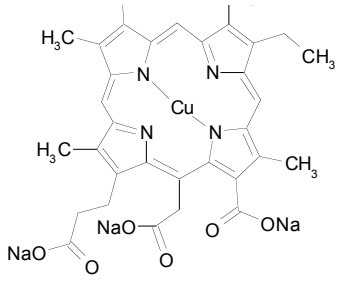
Chlorophyllin (CHL) is a water-soluble, sodium-copper salt of chlorophyll, with potent antimutagenic activity *in vitro* [1]. Preclinical studies showed that CHL inhibited the formation of carcinogen-DNA adducts and tumors in animals treated with heterocyclic amines, polycyclic aromatic hydrocarbons, and aflatoxins (see [2] for a review). When co-administered with these carcinogens, CHL formed molecular complexes and lowered their bioavailability [3-5]. Recognizing that this mechanism also may be applicable in the human situation, a 3-month intervention with CHL protected significantly in a population at high risk for liver cancer [6].



Chemical structures of chlorophyllin and chlorophyll

Post-initiation the situation is more complicated, with preclinical studies demonstrating both tumor suppression and promotion by CHL (see [7] for a review). Thus, attention was turned to natural chlorophylls, which inhibited multi-organ carcinogenesis in the rat and trout [8,9]. In recent studies with human volunteers, and using the exquisite sensitivity of accelerator mass spectrometry (AMS), CHL and natural chlorophyll both reduced the uptake and bioavailability of an ultra-low dose of AFB_1. These findings suggest that a human intervention trial is warranted with natural chlorophyll, or chlorophyll-rich foods such as spinach. Key Words: molecular complexes, anti-cancer mechanisms, green vegetables.

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Keywords: molecular complexes, anti-cancer mechanisms, green vegetables

180 Tomatoes, Tomato Carotenoids, and Prostate Cancer

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Prostate cancer is the most common malignancy in American men and dietary approaches that reduce its risk or delay its progression could have profound impact on public health. Epidemiological and animal studies suggest that consumption of tomato products reduces the risk of prostate cancer. Our research team from the University of Illinois and The Ohio State University has collaborated on a number of NIH-funded projects on this topic including an initial RO1 grant in 1997, a recent RO3 (Producing 14C-Phytoene & Phytofluene for Cancer Research; 1RO3CA112649-A) and a current RO1 grant awarded in 2007 (Tomatoes, Lycopene, and Prostate Carcinogenesis in Mice; 1RO1CA125384-01A1) that have focused upon the impact of bioactive components from the tomato on prostate carcinogenesis. We have utilized cell culture and various animal models and determined that lycopene, the primary tomato carotenoid, is but one of many phytochemicals found in tomatoes that may provide protection from prostate carcinogenesis. Moreover, we also hypothesize that the metabolic products of lycopene and other tomato carotenoids, not the parent molecules, may have biological activity. In our current studies we utilize several murine models to determine if the tomato carotenoids, lycopene, phytoene, and phytofluene, or their metabolic products produced by carotenoid cleavage enzymes, alter the risk of development and progression of prostate cancer. We are utilizing two novel mouse strains that lack one of the two known mammalian carotenoid cleavage enzymes, 15, 15' monooxygenase knock-out (CMO-I KO) and 9', 10' monooxygenase knock-out (CMO-II KO). The current grant addresses three major specific aims. *Specific Aim 1* is to evaluate the tissue-specific expression of CMO-I and CMO-II in A) wild-type, CMO-I KO, and CMO-II KO mice following short and long-term feeding of different levels of tomato powder or lycopene. We will then examine the expression of CMO-1 and CMO-II during prostate carcinogenesis in TRAMP mice. *Specific Aim 2* will precisely determine how changes in CMO-I and CMO-II expression dictate the tissue biodistribution of tomato carotenoids and production of lycopenoids and other tomato carotenoid metabolites. These studies will employ wild-type, CMO-I KO, and CMO-II KO mice. *Specific Aim 3* will evaluate the effect of altered tomato carotenoid metabolism on prostate cancer development by quantitating the ability of dietary tomato powder or lycopene to inhibit prostate cancinogenesis in TRAMP, TRAMP x CMO-I KO, and TRAMP x CMO-II KO mice. Our ongoing studies suggest that lycopene is metabolized primarily by the CMO-II enzyme and not cleaved by CMO-I, which is known to cleave beta carotene. Moreover, we have identified two apo-lycopenal cleavage products in rodent tissues that are of keen metabolic interest. Together, these studies will allow us to determine if tomato carotenoids, or their metabolic products, are able to modulate prostate carcinogenesis and if CMO-I/CMO-II expression defines the response to tomato carotenoids. Our preclincial studies are relevent to human application in several directions. For example, we hypothesize that genetic polymorphisms involved in the metabolism of carotenoids, such as in the genes for CMO-I and II, are critical determinants of the benefits of tomato products during prostate carcinogenesis. Our team of collaborators are uniquely qualified to carry out the proposed studies due to our broad expertise with carotenoids, experience with experimental models of prostate carcinogenesis, access to CMO-I and II KO mice, ability to biosynthesize radiolabeled tomato carotenoids using tomato cell suspension culture, and expertise in translating these findings into human clinical trials.

Keywords: lycopene, prostate cancer, carotenoids

181 Dietary and Lifestyle Determinants of Colon Cancer Recurrence and Survival

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Data from pre-clinical and epidemiologic studies and randomized clinical trials demonstrate that regular use of aspirin and cyclooxygenase-2 (COX-2) inhibitors significantly reduces the risk of colorectal adenoma and cancer. Additionally, increasing evidence suggests that vitamin D may reduce the risk of colorectal cancer; large studies indicate a significant reduction in colorectal cancer risk with increasing plasma levels of 25-hydroxyvitamin D_3 [25(OH)D]. Nonetheless, the influence of these factors on the survival of patients with established colorectal cancer remains virtually unknown.

We therefore prospectively studied aspirin and selective COX-2 inhibitor use among 830 patients with stage III colon cancer enrolled in an NCI-sponsored, randomized trial of post-operative adjuvant chemotherapy (CALGB 89803) who completed a detailed survey of medication use and lifestyle midway through adjuvant therapy and then again 6 months after completion of adjuvant therapy. Compared to those who did not report consistent use, consistent aspirin users experienced an adjusted hazard ratio (AHR) for disease recurrence and/or death (DFS) of 0.48 (95% CI, 0.24-0.99), and regular users of either celecoxib or rofecoxib (4.7% of the cohort) experienced an AHR of 0.47 (95% CI, 0.17-1.28). We also prospectively examined the association between prediagnosis plasma 25(OH)D levels and mortality among 304 participants in the Nurses' Health Study and Health Professionals Follow-Up Study who were diagnosed with colorectal cancer from 1991-2002. Higher plasma 25(OH)D levels were associated with a significant reduction in overall mortality (*P* for trend = 0.02). Compared with the lowest quartile, patients in the highest quartile of plasma 25(OH)D had an AHR of 0.52 (95% CI, 0.29-0.94) for overall mortality and a trend toward improved colorectal cancer-specific mortality (AHR = 0.61; 95% CI, 0.31-1.19).

There is a continued need to improve the outcomes of patients with stage III colon cancer. COX-2 inhibition and vitamin D are not routinely incorporated into the care of colon cancer survivors, and definitive data on efficacy and safety could justify recommendations to all such patients. These observational data on COX-2 inhibition and vitamin D justify testing in a randomized control trial. We therefore are developing a large, placebo-controlled, 2 X 2 factorial trial to examine celecoxib and supplemental vitamin D in conjunction with standard adjuvant chemotherapy (FOLFOX) in 2,100 patients with stage III colon cancer (Cancer and Leukemia Group B Trial 80702).

Finally, within the CALGB 89803 cohort, we are actively assessing the influence of other dietary and lifestyle habits on colon cancer recurrence and survival. Moreover, since the influence of diet and lifestyle on prognosis may depend, in part, on the molecular characteristics of the tumor, we are characterizing somatic events within patient's tumors to assess whether the influence of specific dietary and lifestyle habits (e.g., aspirin, vitamin D) on patient survival varies according to molecular alterations within the cancer (e.g., COX-2 overexpression, *KRAS* mutation, respectively).

Keywords: colorectal cancer, survival, lifestyle habits

182 Chemoprevention of Lung Carcinogenesis Using Green Tea: A Phase IIB Randomized, Double-blinded, Placebo Controlled Trial of Green Tea Extract and Polyphenon E in Former Smokers with Chronic Obstructive Pulmonary Disease (COPD)

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Many laboratory studies have shown an inhibitory action of green tea or the polyphenolic fraction of green tea in animal models of lung carcinogenesis. Thus, the role of tea drinking as a potential inhibitor of carcinogenesis merits careful evaluation. In our attempt at translating the abundant pre-clinical information and epidemiological data to the human population, we are completing a Phase IIb 3-arm randomized, placebo controlled, double blinded green tea intervention trial among former smokers with chronic obstructive pulmonary disease (COPD) and >= 30 pack-years of smoking history. This population is targeted because they have been identified as having a high prevalence of premalignant dysplasia. Subjects will be randomly assigned to consume daily for six months either a standardized green tea (GT) beverage, or a defined green tea polyphenol (GTP) extract in capsule form, or placebo preparations. The hypotheses to be tested in the proposed research are 1) high consumption of GT or GTP can protect against cellular oxidative damage and 2) high consumption of GT or GTP can modulate the expression of genes involved in proliferation and apoptosis in a population at elevated risk of lung cancer. The primary endpoints will be improvement in markers of oxidative damage in DNA and lipid (levels of 8-OHdG, 8-epi-PGF2, and catalase, superoxide dismutase and glutathione peroxidase activities). The secondary endpoints will be exploratory to assess changes in the gene expression of biomarkers of proliferation (EGFR, PCNA, JUN, FOS, Ki-67) and apoptosis (caspase-3) in induced sputum. In addition, we will seek to determine if there are differences in adherence between the green tea preparation groups. We believe that a program of nutritional intervention by realistic dietary modifications that are effective, safe, and acceptable should be the cornerstone of lung cancer prevention strategy.

Keywords: chemoprevention, lung, green tea

183 Dietary Histone Deacetylase Inhibitors for Cancer Prevention: From Cells to Mice to Man

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Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables, such as broccoli and broccoli sprouts. This anticarcinogen was first identified as a potent inducer of Phase 2 detoxification enzymes, but evidence is mounting that SFN acts through various other mechanisms. SFN has been shown to inhibit histone deacetylase (HDAC) activity in human colon and prostate cancer lines, with an increase in global and local histone acetylation status, such as on the promoter regions of *P21* and *bax* genes. SFN also inhibited the growth of prostate cancer xenografts and spontaneous intestinal polyps in mouse models, with evidence for altered histone acetylation and HDAC activities in vivo. In human subjects, a single ingestion of 68 g broccoli sprouts inhibited HDAC activity in circulating peripheral blood mononuclear cells 3-6 h after consumption, with concomitant induction of histone H3 and H4 acetylation. These findings provide evidence that one mechanism of cancer chemoprevention by SFN is via epigenetic modulation of HDACs. Other dietary agents such as butyrate, biotin, lipoic acid, garlic organosulfur compounds, and metabolites of vitamin E have structural features compatible with HDAC inhibition. Pharmacological HDAC inhibitors have shown promise as anti-cancer agents and are currently in human clinical trials. The ability of dietary compounds to de-repress epigenetically-silenced genes in cancer cells, and to epigenetically prime these genes in normal cells, has important implications for cancer prevention and therapy using easily accessible foods with fewer side-effects as compared with potent HDAC inhibitor drugs. Supported by NIH grants CA107693, CA122906, CA090890, CA122959, and by Environmental Health Sciences Center grant P30 ES00210, from the National Institute of Environmental Health Sciences.

Keywords: epigenetics, histone deacetylase inhibitor, dietary chemoprevention

184 Diet, Inflammation and Tumor Formation in the Intestine

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A fully defined rodent diet formulated to mimic, both qualitatively and quantitatively, intake of major risk factors for colon cancer in the United States, accelerates and increases development of intestinal tumors in every mouse genetic model of intestinal cancer in which it has been investigated. As in the human population, this diet is effective in causing colon tumors when fed to wild-type, C57Bl/6 mice for approximately 2/3 of their lifespan (1.5-2 years). This is a model for sporadic colon cancer, responsible for >90% of colon cancer in western countries, and our data indicate this involves effects on gene expression profiles similar to those caused by inherited mutation of the Apc gene.

The risk factors in the western-style mouse diet are higher fat, lower calcium and vitamin D_3, and lower donors to the single carbon pool (folate, methionine, choline and fiber). The diet is formulated to adjust intake levels of these nutrients, on a nutrient-density basis, to reflect levels in the general population linked to elevated incidence of colon cancer. In both the genetic and dietary mouse models of intestinal cancer, elevating calcium and vitamin D_3 to levels demonstrated to be chemoprotective for human colon cancer prevents the increase in tumor formation, therefore identifying these nutrients as likely key components of the human diet that determine relative risk for colon cancer.

The development and progression of colon cancer is clearly associated with inflammation and can be significantly reduced by anti-inflammatory drugs. Since vitamin D_3 has both anti-inflammatory and chemopreventive activity, we investigated the role of inflammation and vitamin D_3 on tumorigenesis. Inflammation was generated in the intestinal mucosa using three mouse genetic models: in $Pofut^{flox/flox}$-villin:cre mice, which inactivates the fucosyltransferase necessary for efficient interaction of all Notch receptors with Delta and Jagged ligands, down regulation of Notch signaling led to pronounced secretory cell metaplasia in the small and large intestine, and to a major inflammatory response involving infiltration of macrophages, and T and B cells, associated with dysplasia that can progress to tumor formation (Guilmeau et al). Marcrophages and cells of the monocyte lineage, were more directly targeted by Lin and colleagues in generating a $Stat3^{flox/flox}$, cfm-cre model, which led to a rapid inflammatory response, and marked tumor formation; finally, Velcich uncovered a low-level inflammatory response in the $Muc2^{-/-}$ mouse she developed, in which the gene that encodes the major gastrointestinal mucin is inactivated and tumors arise throughout the small and large intestine, and rectum, that are increased by the western diet or by introduction of a mutant Apc allele. In each of these 3 models, there was also evidence for an important role of the intestinal microflora in the establishment of the inflammatory response.

Consistent with the particular importance of macrophages in tumorigenesis, Klampfer and colleagues have shown that conditioned media from human macrophage cultures, or co-culture of human macrophages with human colonic carcinoma cells, respectively, increased growth of the epithelial cells through stimulation of Wnt signaling in the epithelial cells. Moreover, elevation of growth and Wnt signaling in the epithelial cells was eliminated by treatment of the macrophages with vitamin D_3 that required expression of the vitamin D receptor by the macrophages, and was due to interleukin 1 signaling from the macrophages. In related work, Wang showed that colonic carcinoma cell growth could also be stimulated by mouse macrophages.

Thus, signals passing among and between mucosal inflammatory and epithelial cells, and the pathways involved, are potential targets for chemoprevention of colon cancer by dietary or pharmacological approaches.

Keywords: diet, inflammation, colon cancer

185 Biomarkers for Caloric Restriction in Rats: Biomarkers for Cancer Risk in Humans?

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Caloric restriction (CR) in rodents is associated with decreased morbidity and increased longevity in rats -- a complement to the observation that obesity is associated with increased morbidity and mortality in humans. The phenotypic effects of CR are remarkably robust. As one example of the power of the CR paradigm, rates of mammary cancer incidence are generally reduced at least 90% in CR animals, fewer tumors are found in these animals, and those tumors found are reduced in size. CR appears to affect initiation, promotion, and progression phases of the disease, and may be dominant against genetic predisposition, specific components of the diet, and specific environmental carcinogens.

We therefore propose that biomarkers that can identify *ad libitum* fed and CR rats with a high degree of accuracy will predict disease risk in humans (ie, by, in part, recognizing a metabolism reflective of resistance to disease). One complication is that the metabolic state characteristic of caloric intake might encompass at least four elements: (i) short term response to lower food intake; (ii) body weight/body mass index; (iii) long-term adaptation to low calorie diets, and; (iv) beneficial physiological effects associated with the response to such diets. The "signal" of such a response may be further confounded by effects across different tissues, times and, in outbred populations, eg humans, in the complex gene/environment (eg diet) interactions.

Omics-based approaches offer a possible means around the limitations induced by complexity. The strategies focus on melding analytical technologies capable of simultaneously querying multiple biochemical compounds/pathways, etc., with computational approaches and workflows capable of finding signal in a large background of noise. Metabolomics and proteomics are well suited to the types of ongoing epidemiological studies.

We have therefore developed serum metabolomic profiles that can identify *ad libitum* fed and caloric-restricted rats with a high degree of accuracy. These profiles have been adapted for use in human epidemiology studies, with several long-term goals, including objective analysis of diet in humans and individualized risk prediction for diseases involving metabolic components, such as breast cancer. Exploratory studies previously identified 93 redox-active small molecules from sera (measured by HPLC coupled with coulometric detector arrays) with potential to distinguish dietary groups in both male and female rats. Classification and predictive power were addressed using a series of megavariate data analysis approaches in both open and blinded analyses across the lifespan and across different extents of nutritive intake. Notably, we found that the use of appropriate algorithms allowed us to distinguish such diets across several years, even when the signal due to caloric intake was apparently swamped by the cohort-cohort differences observed. We have now begun to adapt these marker profiles for use in human epidemiology studies. In particular, we have shown that we can identify the majority of our markers in humans and that these can pass classical tests, such as blinded precision (analytical) tests, stability requirements, and tests examining inter- vs intra-individual variability. We will present the models, their ability to distinguish sera based on caloric intake, and the initial results of tests moving these markers to epidemiological studies in human plasma.

Keywords: caloric restriction, risk, breast cancer

186 Translating Neuroscience, Pharmacology and Genetics to Improve Therapy for Nicotine Dependence

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Nicotine dependence is a complex condition presenting challenges to efforts aimed at identifying its biological underpinnings and development of efficacious medications. Among the many interacting neurobiological systems implicated in nicotine dependence, emerging preclinical and clinical data support the role of the endogenous opioid system. This translational project focuses on the role of the mu opioid receptor in nicotine reward and treatment response. We have shown that pharmacologic and genetic manipulation of the mu opioid receptor (MOR) alters behavioral expression of nicotine reward in rodent models (Walters et al., 2005). The human MOR gene (*OPRM1*) has a well characterized functional polymorphism Asn40Asp wherein the minor allele (Asp40) is associated with altered mRNA and protein levels, and binding affinity. We have also demonstrated that the reinforcing properties of nicotine are attenuated among human carriers of the Asp40 allele (Ray et al., 2006), as are effects of nicotine withdrawal (Lerman et al., 2004; Wang et al., 2008). Most importantly, Asp40 carriers have a greater ease of quitting smoking in clinical treatment (Lerman et al., 2004; Ray et al., 2008). Extending this line of research, Dr. Blendy's laboratory has developed a knock-in mouse that possesses the human Asp40 in the MOR gene, and is currently characterizing the mouse at the molecular and behavioral levels. In parallel, a human positron emission tomography (PET) study is examining MOR binding in smokers, as a function of *OPRM1* genotype and nicotine exposure. The ultimate goal of these translational research studies is to characterize the protective effects of the *OPRM1* Asp40 allele in order to develop medications that target these neurobiological processes for the treatment of nicotine dependence.

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Keywords: prevention, tobacco, neuroscience

187 Fatty Acid Synthase Inhibition for Ovarian Cancer

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Fatty acid synthase (FAS), the enzyme responsible for the *de novo* synthesis of fatty acids, is highly expressed in many human cancers, including ovarian cancer. Prior studies have shown that pharmacological FAS inhibition is cytotoxic to a variety of human cancer xenograft models without overt toxicity. Thus, FAS has become a target for anticancer therapy. While successful xenograft studies suggest that FAS inhibition is not substantially toxic to normal tissues, formal toxicity data has not been described. Utilizing a novel small molecule FAS inhibitor developed by FASgen Inc. FSG-31, in preliminary toxicity studies, we report no observable toxicity to normal tissues in the rat or mouse. FSG-31 inhibits fatty acid synthesis in SKOV3 human ovarian cancer cells *in vitro* (IC_{50} = 6.09 ± 0.4 μg/ml) at a concentration similar to induce cytotoxicity (LC_{50} = 5.2 ± 2.0 μg/ml). FSG-31 (50 mg/kg/day) for 2 weeks substantially inhibited SKOV3 xenograft growth by >90% compared to vehicle. FAS activity in the treated tumor xenografts was reduced by 82%. This is consistent with published *in vitro* studies where inhibition of FAS pathway activity by at least 20% led to brisk apoptosis in human cancer cells. No gross or microscopic organ toxicity was identified. In maximally tolerated dose studies, male and female rats were challenged with increasing single doses of FSG-31 ranging from 0 (vehicle) to 1000 mg/Kg ip or po and followed for 8 days. Aside from one death in the female rats treated with 1000 mg/Kg (ip), no overt toxicity was noted in any other animals; normal motor activity was recorded. In a five-day rat toxicology study, male and female rats were challenged with twice-daily doses of FSG-31 from 0 to 500 mg/Kg orally. No abnormal behavior or distress was noted attributable to FSG-31. The following chemistry and hematology studies were within normal limits: Na^+, K^+, Cl^-, HCO_3^-, creatinine, AST, ALT, ALP, glucose, bilirubin, hemoglobin, hematocrit, white blood cell count and differential, platelets, and coagulation profile. No gross or microscopic organ toxicity was seen. Finally, we conducted pharmacokinetics and bioavailability studies of FSG-31 in rat and dog. In dogs, FAS-31 half-life is between 2.5 h in the fasted state and 3.6 h in the fed state with 16% and 51% plasma bioavailability respectively. In rats, FSG-31 half-life is 5 h in males and 3.8 h in females with 17% plasma bioavailability. Pharmacokinetic and toxokinetic analysis of FSG-31 respectively, in mice and rats treated with doses of FSG-31 used in xenograft models (50 mg/kg), demonstrate blood levels of FSG-31, which if applied to cancer cells, would inhibit FAS pathway by approximately 50%. Thus, the blood levels of FSG-31 achieved in the toxicological studies are sufficient to induce apoptosis in human cancer cells and were non-toxic. In addition, FASgen Diagnostics developed an ELISA assay which has detected high levels of FAS in the serum of cancer patients. This biomarker may aid in monitoring disease progression and selection of patients for FAS therapy. From these studies, we conclude that FAS is a viable pharmacological target for anti-cancer therapy and FSG-31 will progress into clinical development.

Keywords: fatty acid synthase, ovarian cancer, targeted therapy

188 Obesity and Prostate Cancer Progression in the Physicians' Health Study

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Obesity is an epidemic in the United States and globally. The consequences of obesity for prostate cancer (PCa) are considerable, since adiposity is generally linked with poor PCa prognosis, although the mechanisms are unknown. We are undertaking a comprehensive evaluation of obesity and PCa within the prospective Physicians' Health Study (PHS), integrating rich exposure and clinical data with databases on genetic variants, plasma biomarkers and tumor expression.

Since 1982, 3,000 men have been diagnosed with incident PCa, of whom 375 have died of cancer or developed metastases. In the PHS, obesity measured 20-years prior to cancer diagnosis is strongly linked with PCa mortality. Based on the prevalence of obesity in the US, prediagnostic excess body weight could account for 34% of PCa death among men with PCa. We measured prediagnostic plasma levels of two obesity biomarkers: c-peptide (a marker of insulin control) and adiponectin (an adipocytokine). Men with high c-peptide levels, an indication of hyperinsulinemia, had a 2.6 times (95% CI 1.4-4.6) greater risk of PCa-specific mortality compared to men with lower levels, while high circulating adiponectin, inversely related to body mass index, was associated with improved cancer-specific survival (RR 0.40, 95% CI 0.18-0.89).

Obesity with insulin resistance can lead to inactivation of AMP-activated protein kinase (AMPK), a metabolic regulatory enzyme that normally suppresses function of fatty acid synthase (FASN). Inactivation of AMPK may permit upregulation of FASN and downregulation of adiponectin. We measured tumor expression of FASN and adiponectin's receptor 2, and assessed the impact of obesity on cancer survival according to expression of these markers. We found FASN overexpression is associated with an increased risk of lethal PCa for those who were overweight. Moreover, high tumor expression of Adipor2 (which was positively linked with FASN expression) was associated with an almost 3-fold increased risk of PCa-specific mortality, independent of clinical variables.

Adiposity is also linked with lower circulating androgen and higher estrogen levels. Androgens and estrogens affect the prostate epithelium via their respective receptors. We examined whether the effect of obesity on PCa survival differed as a function of tumor expression of AR and ER, and found evidence of a strong interaction between adiposity and AR expression, such that men obese prior to cancer diagnosis were at 5.6 times (95% CI 1.27-24.4) greater risk of lethal PCa if they had high expression of AR. We interrogated tumor expression profiles of 6144 genes among 112 men using Illumina DASL. Men were classified as obese (BMI>27.5) or healthy weight (BMI 18.0-24.9) at baseline in 1982. Gene Set Enrichment Analysis was implemented to search for pathways differentially expressed according to obesity status. Preliminary analyses suggest that obese men had tumors that exhibited dysregulated expression of inflammation pathways: NF-kappa beta, interferon-gamma, and tumor necrosis factor. These data are in line with the well-documented systemic effects of obesity on inflammation, and suggest that obesity even years prior to diagnosis can influence prostate tumor biology.

Taken together, our data in the PHS highlight the complexity of mechanisms involved in the obesity-PCa relationship, and provide supportive evidence of both systemic and tissue-specific effects of excess weight. The findings point to multiple opportunities for intervention.

*** DF/HCC Prostate Cancer Epidemiology Group** (in alphabetical order): Eisenstein A, Finn S, Fiore C, Fiorentino M, Giovannucci E, Golub T, Judson G, Kantoff P, Kasperzyk J, Li H, Loda M, Ma J, Mucci LA, Nguyen P, Penney K, Perner S, Qui W, Rubin MA, Sinnott JS, Stampfer M, Stark JR

Keywords: prostate cancer, obesity, biomarkers

189 Cardiovascular Risk and Response to a 12-Month Behaviorally-Based Lifestyle Intervention Among Young Adult Survivors of Childhood Acute Lymphoblastic Leukemia

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Survivors of childhood acute lymphoblastic leukemia (ALL), the most common childhood cancer, have an increased risk of premature cardiovascular mortality. Aims of the study were to (1) determine the prevalence of insulin resistance and other cardiovascular risk factors in young adult survivors of childhood ALL and compare with population-based controls and (2) determine the effectiveness of a behaviorally-based 12-month *Lifestyle* intervention on physical activity and cardiorespiratory fitness compared with a standard control group.

Of 189 eligible ALL survivors, 62.4% (118/189) agreed to participate in the study. Age, sex, race/ethnicity, age at cancer diagnosis, and interval from cancer diagnosis to study were not significantly different between participants and non-participants. The median age of participants was 23.0 years (range, 18-37); 55.9% were female, and 26.1% racial or ethnic minorities.

A cross-sectional assessment was completed to determine the prevalence of insulin resistance and other cardiovascular risk factors. For perspective, ALL survivors (N=118) were compared to a probability-based cohort of 30-37 year-old individuals from the same region participating in the Dallas Heart Study (DHS, N=782). ALL survivors had a significantly higher homeostatic model for assessment for insulin resistance (HOMA-IR), adjusted for body mass index and race/ethnicity, in comparison with DHS participants ($P<0.001$). Women treated with cranial radiotherapy (CRT) had a significantly increased prevalence of cardiovascular risk factors; they were 6.1 times more likely to multiple risk factors in comparison with DHS women (95% CI 2.2-16.8). Findings in women treated with only chemotherapy and male survivors treated with and without CRT had increased but attenuated cardiovascular risk.

To further investigate ALL therapies associated with risk, we assessed measures of obesity and physical activity/cardiorespiratory fitness. Survivors treated with CRT had higher levels of abdominal and visceral fat and body fat percentage than those who were treated with only chemotherapy. Most survivors were in the lowest NHANES level of cardiovascular fitness (females, 77.2%; males 59.6%). This low level of cardiorespiratory fitness was independently associated with exposure to anthracyclines and to CRT. Notably, in comparison with male survivors, women were significantly more likely to be physically inactive and have a low level of cardiorespiratory fitness.

Of the 118 participants, 66 were physically inactive (less than 150 minutes of moderate or vigorous physical activity per week) and were randomized to a 12-month clinical trial comparing a behaviorally-based *Lifestyle* intervention to a standard control. Among those completing the 12-month measurements (intervention group, 59.4%; control group, 79.4%), HOMA-IR was significantly lower for participants in the intervention group ($P=0.029$). Cardiorespiratory fitness and levels of physical activity energy expenditure were not different between the two groups.

Keywords: leukemia, cardiovascular, radiotherapy

190 Changing Diet Improved Health Outcomes Among Breast Cancer Survivors Without Hot Flashes and May Be Explained by Circulating Estradiol Concentrations

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The WHEL Study was a randomized controlled trial designed to test the hypothesis that a major increase in vegetables, fruit and fiber and decrease in fat would improve prognosis in women with early stage breast cancer., An additional hypotheses was that such dietary change would substantially increase circulating antioxidants and/or reduce circulating estrogen concentrations through the enterohepatic recirculation pathway brought about by an increase in the fiber-to-fat consumption ratio.

Between1995 and 2000, we enrolled 3088 women diagnosed with stage 1 (>1cm) to III (AJCC IV) breast cancer within the past 4 years. On average, study participants were following national recommendations of 5 servings of vegetables and fruits per day when they entered the study. The intervention was associated with a major dietary change maintained through at least 4 years (+65% vegetables, +25% fruit, +30% fiber, -13% energy from fat), resulting in a 43% increase in circulating carotenoids at 4 years. At 4 years, the fiber-fat consumption ratio was 58% higher in the intervention versus the comparison group.

Progression-free survival was assessed semi-annually by telephone and all events were confirmed by medical record review. Study outcome information was available for 96% of women at the close of the study (June 2006). There were 518 confirmed breast cancer events (2/3rds distal recurrence) and 315 deaths (80% from breast cancer). In the overall sample, the dietary change did not reduce either breast cancer events or mortality from any cause versus comparison.

In addition to tumor characteristics, both premenopausal status and the absence of hot flashes following treatment (i.e. at baseline), predicted study outcome. Both or these variables suggest higher baseline circulating estradiol concentrations. Among postmenopausal women, baseline circulating estradiol concentrations predicted additional breast cancer events. In an early sub-sample, the study dietary pattern reduced circulating estradiol concentrations among postmenopausal women. Postmenopausal women without hot flashes who were randomized to the intervention did not have the approximately 30% higher event rate observed in the comparison group.

These findings fit the theory that circulating estradiol concentrations are important to breast cancer growth and indicate that the absence of hot flashes following treatment may be an easily-assessed clinical marker of higher risk in early stage postmenopausal breast cancer patients. The study results suggest that the reduction in risk achieved by the study dietary pattern may have been achieved by lowering circulating estradiol concentrations in the higher risk group.

Keywords: diet, estrogen, health outcomes

191 Micronutrient-Based Approaches for Control of High-Risk Human Papillomaviruses and Their Role in the Prevention of Cervical Intraepithelial Neoplasia

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Micronutrients such as folate, vitamins B-12, A, C, E and carotenoids play important roles as inhibitors of human carcinogenesis in several organs. These micronutrients are particularly important for cancers in the cervix because high risk human papillomavirus (HR-HPV), classified as a human carcinogen plays a causative role in cervical carcinogenesis. Because there is no cure for HPVs, prevention and control of these infections could be used for primary prevention of cervical intraepithelial neoplasia (CIN) which are precursor lesions for cervical cancer (CC), especially when women are already infected with HPVs where HPV vaccines are ineffective or when the vaccines are not affordable or its acceptability is low. We previously reported that higher circulating concentrations of folate are independently associated with a lower likelihood of becoming positive for HR-HPVs and of having a persistent HR-HPV infection, and a greater likelihood of becoming HR-HPV negative (Cancer Res 2004; 64: 8788-93). Our studies have also demonstrated that women with lower folate status and positive for any 13 types of HR-HPV were two times more likely to have higher grades of cervical intraepithelial neoplasia (CIN 2 +). More importantly, we also demonstrated that women with lower folate and positive for HPV-16 were 9 times more likely to have CIN 2 + strongly suggesting a specific effect of folate on HPV 16 (OR=9, 95% CI 3.3, 24.8), the most commonly found and most carcinogenic type of HPV. Biological mechanisms by which folate could modify the risk associated with HR-HPV is highly plausible and include folate's effects on immune response, integration of HPV into host genome via folate sensitive fragile sites, HPV proliferation and methylation of HPV DNA. Currently, we are investigating the effect of supplementation with folic acid on clearance of HPV 16 and other HR-HPV and on preventing the transformation of HPV associated cellular changes to CIN 2+ by conducting a 12-month double-blind, placebo-controlled trial of supplementation with 5 mg of folic acid per day (R01 CA102489-PI, Piyathilake). This study is also designed to generate data on the mechanisms of action of folic acid on the behavior of HR-HPV, which will aid greatly in understanding the mechanisms of action of folate in cervical carcinogenesis.

Keywords: HPV, cervix, folate

192 Identification of Selenium Responders: Who Will Benefit From Selenium Supplentation for Cancer Risk Reduction?

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Pre-clinical studies of selenium supplementation have proven instrumental to elucidate selenium biochemistry, metabolism, and incorporation into selenoproteins Emerging data on the potential anticarcinogenic mechanisms of selenium lend biological plausibility to its chemopreventive potential in humans. These putative mechanisms include protection against oxidative damage, alterations in carcinogen metabolism, stimulation of apoptosis, and inhibition of angiogenesis. Teams of collaborators led by one of us (HJT) conducted two randomized, double blind, placebo-controlled, translational biomarker studies to determine if selenium supplementation influenced markers of selenium bioactivity and/or risk biomarkers for breast cancer and surrogate endpoint biomarkers for lung cancer. Male or female participants (N=123 for lung and N=111 for breast) were randomly assigned to receive either a placebo tablet or a tablet containing 200 μg high-selenium brewer's yeast for a duration of 6 months (lung) or 12 months (breast). Data from these studies will be presented to support discussion about the factors that may determine selenium responsiveness and disease risk reduction. The goal is development of an algorithm that identifies individuals who will respond to selenium supplementation in a manner that reduces their risk for cancer. Areas that will be addressed include: 1) gender differences in selenium responsiveness, 2) mode of selenium action (selenoprotein-mediated, methylated selenium metabolite, or hormetic response, 3) role of genetic polymorphisms in modulating responsiveness, and 4) cancer pathway specificity of selenium action.

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Keywords: biomarkers, selenium, cancer risk

193 An Epidemiological Study of Acquired Mutations and Epigenetic Changes in Colorectal Cancer

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Colorectal cancer is a heterogeneous disease as can be seen by the differences in risk factors often observed by sex, tumor site and age. In this study we further evaluate the heterogeneity of the disease by examining acquired genetic and epigenetic alterations that exist in colorectal tumors and the unique characteristics associated with those changes in the population. We evaluated mutations in the *p53* and Ki-*ras* genes and microsatellite instability (MSI) and CpG Island Methylator Phenoytpe (CIMP). Using data from a large population-based study of colorectal cancer cases from three centers in the United States, we evaluate the impact of mutations and epigenetic changes on survival and how specific lifestyle risk factors are associated with specific mutations.

In our study population of over 2000 tumors, we observed that tumor occurrence was more similar for distal and rectal tumors than for proximal tumors. The major contributing factor to the difference is a much higher frequency of MSI and CIMP in proximal tumors. MSI tumors were associated with improved survival of proximal and distal tumors, although decreased survival for rectal tumors. Ki-ras mutated and CIMP positive tumors were associated with worse survival among those with distal and rectal tumors.

Assessment of lifestyle factors associated with tumor markers suggests unique associations with various markers. Cigarette smoking increased the likelihood of having a MSI and a CIMP positive tumor. Higher levels of alcohol intake were associated with MSI tumors and CIMP negative tumors. High levels of BMI were associated with Ki-ras mutations. Insulin-related genes appear to influence risk of developing a CIMP positive tumor.

Taken together our data suggest colorectal tumor mutations, while occasionally overlapping, most often represent unique disease pathways. Evaluation of environmental, genetic, and lifestyle factors in conjunction with tumor markers has given insight into development of these pathways. A better understanding of cancer development provides a basis for cancer prevention and treatment.

Keywords: colorectal cancer, CIMP, MSI

194 Coupling Dieting for Weight Loss and Chemoprevention: Can Intermediary Metabolism Be Targeted to Use Cancer's Achilles Heel for Prevention That Is Sustained in the Absence of Continuous Treatment?

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Emerging evidence from studies of human populations indicates that excessive energy intake is associated with an increased risk for cancer, but that that dietary energy restriction is protective. Similarly, dietary energy restriction has been shown to be a potent inhibitor of carcinogenesis in most of the experimental models in which it has been investigated. However, current trends in the prevalence of overweight and obesity suggest that simply recommending limits to daily energy intake will be neither feasible nor attractive to most individuals as a cancer prevention strategy. It was for this reason that in our current work we decided to determine if it is possible to identify drugs that mimic the cancer preventive activity of dietary energy restriction in the absence of limiting energy intake; and the answer is yes, with metformin being an excellent example of such an agent. But ongoing work goes beyond this objective. Our goal is to define a strategy for cancer prevention in which short term treatment with a pharmacological agent can be combined with a bout of dietary energy restriction associated with weight loss and that would result in sustained protection against cancer in the absence of continuous intervention. Multiple points of attack on intermediary metabolism and ATP generation are being investigated, an effort directed by the metabolic reprogramming that has been documented to occur in cancers at many organ sites. The ability to sustain protection against cancer by the selective deletion of transformed cells via apoptosis would represent a major advance in efforts to prevent and control the occurrence and recurrence of this disease.

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Keywords: dieting for weight loss, chemoprevention, breast cancer risk

195 Discovery and Validation of Molecular Targets, Biomarkers, and Nontoxic Interventions for Cancer Prevention

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The Gene Regulation Section, Laboratory of Cancer Prevention (LCP) is focused on understanding the early events in tumorigenesis with the hope of finding new molecular targets, biomarkers and non-toxic interventions that will prevent or significantly delay the beginning of cancer. Our work in vitro and with genetically engineered mice (GEM) has repeatedly shown that the transcription factor AP-1 can be targeted for cancer prevention and intervention (Young et al., 2003). Specifically, mice that express dominant negative c-Jun (TAM67) are protected from environmental and oncogene induced skin and breast cancer without any noted toxicity to the animals (Young et al., 2003, Shen et al., 2008). From these studies we designed a high-through put screen of the NCI-Synthetic and Natural products libraries to identify compounds that would target induced AP-1 activity without affecting cell proliferation (Ruocco et al., 2007). One such compound, NCI676914, although it inhibits AP-1 activation shows greater potency for inhibiting NF-κB activation with an IC50 of about 4 μM (Kang et al., submitted). NCI676914 reduced phosphorylation of IKKα/IKKβ significantly, resulting in a complete block of IκB-α phosphorylation. Concentrations of NCI676914 as low as 1.1 μM repressed NF-κB DNA binding and transcriptional activation of NF-κB dependent genes IL-6 and COX-2. Moreover NCI676914 inhibits tumor promoter induced transformation of JB6 cells and invasion of breast cancer cells. These results suggest that single digit micromolar-range treatment is sufficient to inhibit NF-κB transactivation required for tumor promotion and progression. High selectivity and low toxicity are valuable characteristics of this potential inhibitor against its target. In addition to identifying small molecule inhibitors of AP-1 or NF-κB, the LCP has established multiple collaborations to identify dietary factors that are beneficial for cancer prevention or intervention. The Legume Intervention Feeding Experiment (LIFE) is a collaboration with Pennsylvania State University and Texas A & M. This study is designed to identify indicators of efficacious response to bean-based diets in a clinical feeding study as well as in the laboratory with mouse models of colon carcinogenesis. We have previously shown that a high bean intake is inversely associated with advanced colorectal adenoma recurrence (Lanza et al., 2006). Moreover, mice fed a diet supplemented with navy beans or bean extracts were protected from chemically induced colon carcinogenesis (Bobe et al., 2008). Proteomic analysis of sera from these mice indicated one or more proinflammatory cytokines are reduced in the mice fed the bean-based diets (Mentor-Marcel et al., submitted). In order to identify molecular targets and biomarkers of efficacy, the LIFE study designed a feeding trial in which men at high risk for colorectal cancer were fed bean-based diets for 4 weeks followed by washout and 4 weeks of an isocaloric "American" diet. Serum and fecal colonocytes were collected before, during and after each diet period. Colonoscopy was performed before entering the study. We are analyzing the data collected from these studies.

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Keywords: drug discovery, dietary intervention, prevention

196 Mammary Progenitor Cells as Targets of Dietary Prevention of Breast Cancer

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Breast cancer prevention has to start with the identification of targets and/or processes involved in the initiation of this disease. It has been proposed, but not yet proven, that breast cancer risk is determined in part by the number of susceptible breast stem/progenitor cells that can serve as targets for malignant transformation. To test this idea we are determining whether early life dietary exposures which increase later breast cancer risk also increase mammary progenitor cell population and/or reduce their ability to undergo differentiation to luminal and/or myoepithelial cells, and whether exposures which reduce breast cancer risk reduce mammary epithelial cell proliferation and shift the mammary stem cell hierarchy towards a differentiation pathway. Maternal exposure to a birth weight increasing high fat diet increases, whilst prepubertal exposure to genistein in soy reduces later breast cancer risk. The links between high birth weight or pubertal genistein exposure and altered breast cancer risk have been confirmed both in human populations and animal models.

Our results indicate that maternal exposure to a high fat diet during pregnancy increases offspring's mammary epithelial cell proliferation and the number of terminal end buds (TEBs, which are the sites where malignant transformation takes place). The studies also show that prepubertal genistein exposure reduces mammary epithelial cell proliferation and the number of TEBs. These changes suggest alterations in stem cell behavior. Therefore, we have explored whether the early life dietary exposures affect tumorigenesis in mice that exhibit alterations in stem cell behavior: heterozygous Brca1$^{+/-}$ and Pten$^{+/-}$ mice. Brca1 is enriched in luminal mammary cells and proposed to induce estrogen receptor (ER) positive luminal cell differentiation. Pten is expressed in myoepithelial and luminal cells and involved in controlling stem and progenitor cell pool. Our findings indicate that Pten$^{+/-}$ mice exposed to a high fat diet *in utero* develop more mammary tumors than control diet fed Pten$^{+/-}$ mice. In addition, wildtype mice fed 500 ppm genistein during prepuberty exhibit lower mammary tumor incidence than mice fed 0 ppm genistein. However, prepubertal genistein exposure does not reduce mammary tumorigenesis in Brca1$^{+/-}$ mice. We also found that Brca1$^{+/-}$ mice exhibit a 3-fold reduction in mammary Pten expression, suggesting that lowered Brca1 and Pten expression impairs genistein's ability to prevent mammary tumorigenesis. We next investigated whether the mammary stem/progenitor cell populations are altered in the Pten$^{+/-}$ mice. For that purpose, primary cultures of mammary epithelium prepared from 2-month-old Pten$^{+/-}$ and wildtype mice (n=3 in each group) were characterized by fluorescence activated cell sorter (FACS) for stem/progenitor cell markers Sca-1, CD24, and CD29. The results indicated that stem/progenitor cell populations were not different between Pten$^{+/-}$ and wildtype mice, whist the populations of differentiated luminal and myoepithelial cells were reduced in Pten$^{+/-}$ mice (Table 1).

Table 1.	**Stem/progenitor cells** *CD24^{+}CD29^{high} CD24^{+}Sca-1^{+}*	**Luminal progenitors** *CD24^{+}CD29^{low}*	**Luminal cells** *CD24^{high}CD29^{low}*	**Myoepithelial cells** *CD24^{low} Sca-1^{+}*
Pten+/-	0.09±0.03, 8.5±0.5 3.4±0.3	3.4±0.7	24.7±1.1	3.3±0.2
Wildtype	0.14±0.3, 8.9±0.3 4.2±0.6	4.6±0.4	38.9±3.3	4.7±0.4
	No differences	No differences	P<0.05	P<0.09

The results obtained in this study suggest that down-regulation of Pten leads to a reduction in differentiated luminal and perhaps myoepithelial cell population. Since ER-α is expressed only in luminal epithelial cells, the present findings are consistent with a significant reduction in mammary ER-α content we have previously noted in Pten$^{+/-}$ mice. We are now exploring whether the breast cancer protective effect of prepuberal genistein exposure, which up-regulates Brca1 and Pten, is linked to reduced stem/progenitor cell population and increased epithelial cell differentiation utilizing Brca1$^{+/-}$ and Pten$^{+/-}$ mice. This work was supported by U54CA100970.

Keywords: prevention, mammary stem/progenitor cells, early life dietary exposures

197 Polymer Micelles: From Bench to Bedside

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Polymer micelles have emerged as a new generation of polymer therapeutics for delivery of drugs, genes, and imaging molecules. Various polymer micelles were evaluated in several clinical trials as carriers for anticancer drugs. In particular, Doxorubicin (Dox) incorporated in mixed micelles of Pluronic block copolymers, SP1049C, was the first polymer micelle system to reach clinical evaluation. SP1049C has shown promise in Phase II study in patients with advanced adenocarcinoma of the oesophagus. Pluronics (copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), PEO-*b*-PPO-*b*-PEO) are very potent sensitizers of multidrug resistant (MDR) tumors. Our studies suggest that Pluronic hydrophobic PPO chains immerse into cell membrane micro-domains, called lipid rafts, and induce "membrane fluidization". This is accompanied by inhibition of the ATPase activity of the ATP-dependent drug efflux transport protein, P-glycoprotein (Pgp), overexpressed in MDR cells. Pluronic molecules also internalize into cells via caveolae and ultimately reach mitochondria. This results in 1) depolarization of the mitochondria membrane, 2) inhibition of respiration and 3) depletion of ATP necessary to sustain the Pgp function. Therefore, combined inhibition of Pgp ATPase activity and ATP depletion results in a shut-down of the efflux system and enhanced entry of the drug into MDR cells. The increased delivery of a Pgp substrate, ^{99}Tc-sestamibi formulated with Pluronic in MDR solid tumors was demonstrated in animal models using 1) animal single photon emission computed tomography (A-SPECT) and 2) tumor tissue radioactivity sampling. The phosphorus magnetic resonance spectroscopy (^{31}P-MRS) has shown that after the injection of Dox-Pluronic formulation in tumor-bearing mice the ATP levels and pH in MDR solid tumors drop dramatically while inorganic phosphate (Pi) increases. In addition to that, Pluronic® enhances drug-induced pro-apoptotic signaling in MDR cells and solid tumors. The molecular targets for Pluronic® in mitochondria of MDR cells include respiratory chain Complexes I and IV, which are inhibited by Pluronic. Impairment of mitochondrial function results in accumulation of reactive oxygen species (ROS) and release of cytochrome C, which contributes to cell death via apoptosis. Notably, Pluronic displays remarkable selectivity with respect to MDR tumors phenotype *in vitro* and *in vivo*. For instance, the ATP depletion in cells induced by Pluronic correlates with levels of expression of Pgp. This provides initial evidence that Pgp may be used as a gene expression marker for responses to SP1049C in chemotherapy of cancer. Furthermore, Pluronic® was shown to prevent development of MDR phenotype in breast cancer and leukemia, which can be also used to increase clinical outcomes of SP1049C-based chemotherapy. All together formulation of drugs with Pluronic® represents novel and promising strategy for therapy of drug resistant cancers. Support by the United States National Institutes of Health grant RO1 CA89225 is acknowledged.

Keywords: multidrug resistance, block copolymer, doxorubicin

198 MR Metabolic and Physiological Imaging as Non-invasive Biomarkers for Evaluation of Patients With Glioma

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Purpose: To determine which non-invasive MR imaging biomarkers are of interest for characterizing untreated gliomas with different histological grades in order to determine whether they can contribute to patient management by predicting outcome and assessing response to focal therapy.

Methods: A total of 138 patients with untreated glioma were studied with diffusion tensor imaging (DTI), perfusion weighted imaging (PWI) and 3-D H-1 spectroscopic imaging (MRSI) prior to surgical resection. Histological diagnosis indicated that there were 56 patients with GBM, 37 patients with grade III glioma (21 AAs, 13 OA3, and 3 OD3) and 45 patients with grade II glioma (14 AC, 12 OA and 19 OD). Non-enhancing and where appropriate enhancing and necrotic regions were delineated and applied to maps of relative cerebral blood volume (rCBV), apparent diffusion coefficient (ADC) and fractional anisotropy (FA). Choline (Cho), creatine (Cr), N-acetylasparate (NAA), lactate (Lac) and lipid (Lip) were assessed. Differences between the parameters were assessed for different tumor grades and for grade II and grade III lesions, for different histological sub-types. For the patients with GBM, the MR parameters were compared with survival in order to determine which parameters were predictive of poor outcome.

Results: For patients with grade II gliomas, the ADC, FA and CBV values were the most effective in separating patients with OD versus AC. The median ADC, for example, was 1.57 for ODs, 1.89 for OAs and 2.07 for ACs relative to values in contralateral normal appearing white matter. By using the distributions of ADC values in OD and AC to generate color maps of the probability of values within lesions being characteristic of each tumor sub-type, it was possible to indicate which lesions were of mixed sub-type and to direct tissue sampling to the region of the tumor that would be able to give the most definitive diagnosis. Metabolite levels were highly variable in the grade II gliomas and, while they did help to define the spatial extent of the lesion, were not able to distinguish between different sub-types.

For patients with grade III gliomas, 44% of the lesions that were non-enhancing and in these cases the location of the region with the highest Cho to NAA index (CNI) appeared to be the most malignant region of the tumor. The CBV was relatively low and the ADC values for these grade III lesions was more similar to the values in their Grade II counterparts than in the patients with GBM.

Median anatomic volumes for pre-surgery patients with GBM were CEL 15.1 ± 14.1cc, NEL 43.4 ± 33.5cc and NEC 3.46 ± 8.5cc. Lesions that had a large percentage of the overall T2 hyperintensity that was enhancing or necrotic were correlated with worse survival ($p=.026$, $N=56$, censored=15). Poor survival was also associated with number of voxels with $CNI > 2$ and the volume within the T2 hyperintensity with $nADC < 1.5$. Low ADC in the CEL or high lipid and high lactate within the volume that had $CNI>2$ also predicted a worse survival.

Conclusion: The pre-surgery ADC and CBV parameters were valuable for predicting tumor sub-type in grade II gliomas and may be valuable for directing biopsy or surgical resection. The CNI values were valuable for defining the most malignant region of grade III gliomas, whether or not the lesion was enhancing on T1-weighted MR images. For the patients with GBM, the values of ADC, levels of CNI, Lac and Lip were indicative of more malignant behavior. These variables are thought to correspond to high cell density and regions of hypoxia or necrosis. The information provided by these non-invasive metabolic and physiological imaging data is likely to be important in defining non-invasive biomarkers for stratifying patients to specific treatment protocols and for planning focal therapy.

Keywords: MR imaging, glioma, survival

199 Particle Replication in Non-Wetting Templates (PRINT): Designing Organic Delivery Vehicles for Probing and Treating Biological Systems

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A novel method for the fabrication of organic particles on the order of tens of nanometers to several microns will be described. Our imprint lithographic technique called **PRINT**™ (**P**article **R**eplication **I**n **N**on-wetting **T**emplates), takes advantage of the unique properties of elastomeric molds comprised of a low surface energy perfluoropolyether network, allowing for the production of monodisperse, shape-specific nanoparticles from an extensive array of organic precursors. This engineered nature of particle production has a number of advantages over the construction of traditional nanoparticles such as liposomes, dendrimers, and colloidal precipitates. The nature of PRINT™ technology takes drug delivery for the first time into the uncharted realm of engineered drug therapies given its *á la carte* approach and versatility. PRINT™ allows for the precise control over particle size, shape, composition, cargo, modulus and surface properties and the independent design of these attributes to create truly engineered drug therapies. For the first time, key therapeutic parameters such as bioavailability, biodistribution, and target-specific cell penetration can be simultaneously designed into a therapy or imaging agent. Preliminary *in vitro* and *in vivo* studies are being conducted to demonstrate the promise of PRINT™ particles as delivery vectors and novel imaging agents for the treatment and diagnosis of disease and results from these studies will be presented.

A series of particles with varying sizes, and shapes at a constant chemical composition (i.e. at a constant surface charge) were designed to study the effect of those changes on their internalization by human cervical carcinoma epithelial (HeLa) cells. These findings suggested that cellular internalization of these PRINT particles exhibited a strong dependence on particle size, shape and surface charge. To more clearly delineate the role of specific endocytotic pathways involved in PRINT particle cellular internalization, HeLa cells were treated with known biochemical inhibitors of energy-dependent processes, clathrin-mediated processes, caveolae-mediated processes, and macropinocytotic endocytosis. All of these experiments strongly suggest that clathrin-mediated, and caveolae-mediated endocytosis and to a much lesser extent macropinocytosis are involved with both the nano- and microparticles internalization, but these mechanisms play a larger role with the internalization of the smaller 150 nm (AR = 3) and the 200 nm (AR = 1) PRINT nanoparticles.

A family of three silane crosslinkers has been designed that can be incorporated into the PRINT particles. Cationic PEG hydrogel particles have been fabricated using the dimethyl (DMS), diethyl (DES) and diisopropyl (DIS) silane crosslinkers and with 10 wt% docetaxol as the cargo. The particles were dosed on SKOV3 cells and the efficiency of the cargo release tested using an in vitro MTS assay examining the cell viability. Preliminary results suggest that the diisopropyl silane crosslinked particles were the most efficient at delivering docetaxol and inducing cell death. At 200 μg/mL of particles the cell viability was similar for both the docetaxol control and the DIS crosslinked particles. Additional studies will be required to confirm and understand the results.

The binding and internalization of particles functionalized with antibodies, small molecules and peptides have been studied in a variety of cell lines using flow cytometry, as well as confocal microscopy and transmission electron microscopy. Folic acid and folate receptor mAb functionalized particles were studied using several human cancer cell lines, HeLa, SKOV3 and OVCAR3. The negatively charged particles are not internalized to a great degree even at high particle concentrations whereas the folate functionalized and the positively charged particles are robustly internalized. Similar results were observed for particles functionalized with the mAb to the folate receptor and exposed to SKOV3 cells. Particles functionalized with the synthetically generated peptide to target the Sup-B8 (Burkitts B cell lymphoma) surface sIg were also investigated. Three different densities of targeting peptide were tested and all three densities showed dose dependent internalization by Sup-B8 cells but not into Ramos cells which do not express the Sup-B8 surface sIg. The MTS assay at 96 hours post treatment revealed that the particles with the highest density of targeting peptides were able to induce cell death. The targeting peptide has been reported to cause crosslinking of sIg on Sup-B8 cells and induce cell apoptosis. Further studies are underway to confirm these results. Particles were conjugated with mouse anti-human transferrin receptor monoclonal antibody via biotin avidin coupling. PRINT particles functionalized with TfR

mAb show efficient targeting (63-98% of cell uptake) on seven human cancer cell lines employed in this study (HeLa, Sup-B8, Ramos, H125, SKOV3, MGR3, LNCaP) compared with particles functionalized with TfR mAb isotype control IgG1. Particles decorated with the isotype control IgG1 show little to no binding and internalization on mouse embryonic fibroblast (MEF) which was used as negative control cell line. MEF does not bind isotype control mouse IgG1. The observation of specific internalization for the human cancer cell lines and little internalization for a non-human cell line MEF strongly suggests specific TfR-mediated binding and internalization. Further studies will also include normal human cell lines, which express low level of transferrin receptor, to confirm the specificity of this targeting strategy.

Based on 72 hr MTS assays, the nanoparticles coated with TfR mAb or IgG1 show little cytotoxicity on all the eight cell lines employed in this study except Ramos cells. Approximately 80% of the Ramos cells were killed by TfR mAb functionalized nanoparticles at a concentration of 100 μg/mL. In contrast, free TfR mAb does not induce Ramos cell death. It has been reported that IgA (dimer) or IgM (pentamer) forms of TfR mAb can effectively trigger apoptosis of lymphoma/leukemia cells through suggested mechanism of crosslinking cell surface TfRs, while the IgG (monomer) form of TfR mAb cannot. Similar to the results obtained for the Sup-B8 targeting project described above, surface crosslinking induced cell death is common in B-cell lymphomas, which could possibly explain why TfR mAb coated particles killed only Ramos cells but not the other cell lines tested in this study. Ramos cell viability was also studied as a function of TfR mAb density on particle surface. There was a strong correlation between TfR ligand density on particle surface and cell viability. Investigations into the mechanism of cell death are underway. It may involve surface receptor crosslinking (TfR mAb coated particles have thousands of copies of antibody per particle). It is also possible that cell death is due to depletion of TfR on the cell surface as a result of improper receptor recycling. Regardless of the detailed mechanism, these TfR mAb coated PRINT particles are promising immunotherapeutic agents for B-cell lymphoma and a series of in vivo experiments have been planned.

Keywords: nanomedicine, nanoparticles, PRINT

200 Preliminary Results of a Phase I Study Using Intravesical Administration of Adenoviral-Mediated Interferon-α for Patients With Transitional Cell Carcinoma of the Bladder

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Background: BCG is currently the gold standard for the treatment of recurrent superficial bladder cancer. Despite BCG and other second line therapies, recurrence is a significant problem. Intravesical gene therapy is under investigation as a viable treatment modality due to direct contact between vector and tumor, isolation from vital organs, and easy access to urine and tissue to monitor therapy effects.

Methods: A Phase I, non-randomized, dose escalating, two-center, open label intravesical gene therapy study for BCG refractory superficial bladder cancer patients with CIS/pTa or pT1 disease who refuse cystectomy. Intravesical administration of Ad-IFNα (75ml) with 5 dose levels ($3x10^9$, $1x10^{10}$, $3x10^{10}$, $1x10^{11}$, and 3×10^{11} particles/ml) is planned. 1mg/ml of Syn3 is used as an excipient. Pre-treatment, 0-24 hour, 24-48 hour and 1st voided urine specimens on days 3-7, 10, 14, 28 are tested for IFNα by ELISA. The primary endpoint is safety and tolerability. A secondary endpoint is IFN production detected in urine. The results from the first 11 patients will be presented.

Results: Initial urinary urgency and failure to retain the intravesical instillation for a full hour was present in the first two patients treated with Ad-IFNα/Syn3. Patients 3-11 were pretreated with anticholinergics prior to instillation with minor immediate and no prolonged urinary urgency. Urinary IFN levels (determined at MDACC) for patients receiving $3x10^9$ Ad-IFNα were below the lower limit of the assay (156pg/ml). Peak interferon levels for groups 2-4 were 1038, 10568, and 4689 pg/ml, respectively. Urinary interferon values remain elevated above baseline for 4-7 days. Three patients have had clinical responses and have received a second intravesical instillation.

Conclusions: Preliminary results demonstrate that intravesical administration of Ad-IFNα/Syn3 is safe and well tolerated. Dose levels of $1x10^{10}$ particles are able to initiate prolonged production of urinary IFNα. Continuing studies with higher doses will establish the safety and efficacy of this treatment.

Keywords: bladder cancer, gene therapy, interferon

201 Late Stage Clinical Development of Gene Therapy Approach for Prostate Cancer

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Henry Ford Health System

For the past 15 years our research program has been developing a gene therapy-based approach to treat cancer. Our approach utilizes an oncolytic, replication-competent adenovirus to deliver a pair of therapeutic suicide genes to tumors. The oncolytic adenovirus replicates in, and selectively kills, malignant cells. The suicide gene therapy provides a local chemotherapeutic effect and sensitizes tumor cells to ionizing radiation.

We have successfully translated our approach into the clinic resulting in 6 clinical trials in prostate and pancreatic cancer, including a recently opened randomized, controlled phase 2/3 trial in newly-diagnosed, intermediate-risk prostate cancer. A second randomized, controlled phase 2/3 trial in locally recurrent prostate cancer is being planned for 2008. Our research team, which is comprised of about 12 people (scientists, physicians, technical/clinical staff), conducts all phases of product development including preclinical efficacy and toxicology testing of our products, preparation and submission of required documents (INDs, protocols) to federal agencies (FDA, NIH/RAC) and institutional committees (IRB and IBC), and execution of our clinical trials. During the past 10 years, we have sponsored 4 Investigational New Drug (IND) applications to the FDA, and we currently have 2 products in the clinic.

Our work has been supported almost entirely by NCI-sponsored investigator-initiated grants, including an active Program Project Grant (P01) entitled “Molecular Gene and Radiation Therapies for Cancer”. The current P01 grant supports two preclinical projects, one clinical project that proposed three phase 1/2 clinical trials, and four cores. The projects and cores interact synergistically, making the whole greater than the sum of its parts. The vast majority of the proposed work has been completed and has now progressed into late stage clinical development. Our randomized, controlled phase 2/3 trials contain both clinical and molecular endpoints, the latter of which are designed to test specific hypotheses regarding the molecular basis for the previously observed clinical activity. We hope our clinical trials demonstrate “proof of concept” and generate new knowledge that will foster the development of future products.

Keywords: gene therapy, radiation therapy, clinical trials

202 Targeted Drug Delivery to Tumor Vasculature by a Carbohydrate-Mimicry Peptide

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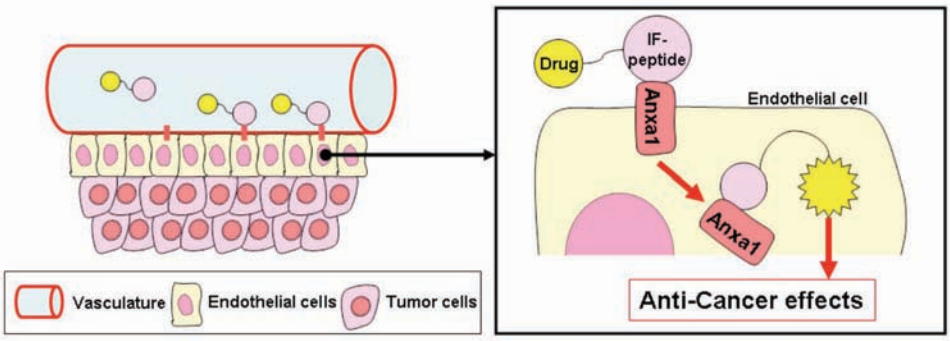
Substantial evidence suggests that cell surface carbohydrates such as sialyl Lewis X (sLex) antigen or selectin ligand promote cancer metastasis. To investigate mechanisms underlying sLex-dependent B16 melanoma colonization of the lung, we screened a peptide-displaying phage library by monoclonal anti-carbohydrate antibodies and identified IELLQAR (I-peptide), which functions as a selectin ligand carbohydrate. I-peptide bound to an endothelial cell surface receptor, inhibiting cell surface carbohydrate-dependent cancer colonization to the lung. Interestingly, I-peptide inhibited lung colonization of sLex-positive B16 cells in E-/P-selectin doubly deficient mutant mice, leading us to propose the existence of a novel carbohydrate-binding endothelial receptor distinct from E-/P-selectins (Zhang et al., Cancer Res 62: 4194-4198, 2002). A presumptive endothelial I-peptide receptor was purified by I-peptide affinity chromatography. Proteomic analysis identified it as an alternative mRNA splicing factor (Sfrs). Recombinant Sfrs protein, expressed in bacteria, bound to a series of fucosylated oligosaccharides in a glycan array. When anti-Sfrs antibody was injected intravenously into mice, lung colonization of carbohydrate-dependent B16 cells was inhibited. These results suggest that Sfrs is responsible for carbohydrate-dependent cancer cell lung colonization.

We also found a fragment of annexin 1 (Anxa1) in I-peptide affinity purified proteins. Since Anxa1 was shown as an endothelial surface marker in tumor vasculature (Oh et al., Nature 429: 629-635, 2004), we screened phage clones displaying an I-peptide-related sequence and identified the peptide that specifically targets tumor vasculature. Thus IFLLWQR or IF-peptide binds Anxa1 with high affinity in tumor vasculature. Upon intravenous injection, chemically synthesized and fluorescence-tagged IF-peptide rapidly accumulated in the tumor and surrounding microvasculatures. Furthermore, when an apoptosis-inducing drug, a geldanamycin analogue was conjugated with IF-peptide, and injected intravenously into a tumor-bearing mouse, tumor growth was suppressed without adverse side effects (see figure).



Chemotherapeutics injected intravenously to a cancer patient are diluted and therefore must be administered to cancer patients at high dose, which can cause systemic side effects. As IF-peptide exhibited the efficacy for chemotherapy targeted to tumors in the mouse, this method should be developed to targeted chemotherapy for human cancer patients. Supported by NIH grant P01CA071932. Glycan array was provided by Consortium for Functional Glycomics

Keywords: tumor-associated carbohydrate antigen, carbohydrate-mimicry peptide, targeting tumor vasculature

203 Lung Cancer Treatment With Pulmonary Aerosol Drug Delivery System

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A wide variety of preclinical studies have indicated that aerosol delivery of chemotherapy agents directly to the lungs holds great potential as a treatment modality, both for lung cancer and for lung metastases of other cancers. We have designed an aerosol delivery system to be easy to use in a hospital, clinical, and possible home environment. The new system has been constructed with computer controlled user-definable drug delivery parameters, a touch-screen control panel, and a computer controlled aerosol delivery system. To make it safe, we have computerized control over aerosol exposure time, dilution air flow rate, aerosol generation, and safety interlocks dependent on setpoints for CO_2 concentration, outlet temperature, flow rate, pressure, oxygen concentration, etc.

At the heart of our system is a special aerosol generator for inhalation delivery of cancer therapy drugs with small, narrow, variable, particle size range and high drug aerosol mass cloud densities. The latest improvements in our aerosol generator will allow us to build a very reliable system for commercial production. To test the effectiveness of the system, we have worked with collaborators at Colorado State University to develop a suitable orthotopic mouse model of nonsmall cell lung cancer using the human lung cancer cell line A549 stably transfected with the luciferase gene to allow the imaging and monitoring of lung tumor formation in live mice. All the *in vivo* studies at CSU will be performed on the improved system for commercial production so that data collected in this project can be used in applying for FDA approval of the system in the future. Data will be presented on the mouse model and on the delivery of aerosolized paclitaxel to mice by inhalation.

Keywords: lung, cancer, aerosol

204 Combined Transductional Untargeting/Retargeting and Transcriptional Restriction Enhances Ad-Mediated Gene Targeting and Therapy for Hepatic Colorectal Cancer Tumors

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Adenovirus vectors have a number of advantages for gene therapy. However, because of their lack of tumor tropism and their preference for liver infection following systemic administration, they cannot be utilized for systemic attack on metastatic disease. For systemic gene delivery, Ad must be "untargeted" from normal cells and re-targeted to tumor cells. Many epithelial tumors (e.g., colon, lung, breast) express carcinoembryonic antigen (CEA).To block the natural hepatic tropism of adenovirus and to "retarget" the virus to CEA-expressing tumors we used a bi-specific adapter protein, sCARhMFE, fusing the coxsackie/adenovirus receptor (sCAR) ectodomain with a single-chain anti-CEA antibody (MFE-23). sCAR-MFE untargets adenovirus-directed luciferase transgene expression in the liver by more than 90% following systemic vector administration. Moreover, sCAR-MFE "retargets" adenovirus to CEA-positive epithelial tumor cells in cell culture, in subcutaneous tumor grafts, and in hepatic tumor grafts.

The Ad fiber-knob is a trimer, suggesting adapter trimerization may enhance tumor retargeting. Trimeric sCARfMFE substantially increases Ad-adapter interaction, enhances CAR-dependent untargeting and enhances CEA-dependent tumor cell infection. Although soluble CEA (sCEA) blocks monomer sCARhMFE-directed CEA-dependent infection, trimer sCARfMFE mediated infection is resistant to sCEA competition. Trimer is also substantially more effective for untargeting adenovirus liver infection following systemic administration and for retargeting virus to hepatic CEA-positive colorectal cancer (CRC) xenografts; sCARfMFE trimerization provides a major advance in Ad transgene gene delivery to hepatic CRC metastases.

Cyclooxygenase-2 (COX-2) is not expressed in liver, but is expressed constitutively in many epithelial tumors. COX-2 transcriptional restriction increased Ad-mediated transgene expression in hepatic tumor xenografts and reduced expression in liver, following systemic virus administration. Utilizing a therapeutic transgene (HSV1-tk) and its prodrug (ganciclovir), COX-2 transcriptional restriction can enhance the therapeutic efficacy, reduce side effects of the therapeutic treatment, and reduce innate immune cytokine responses following systemic Ad vector administration. Combining trimeric sCARfMFE-mediated hepatic transductional tumor transduction with COX-2 restricted transduction increased the efficacy of Ad HSV1-tK/GCV therapy, allowing a lower virus dose to be used to kill hepatic colorectal cell carcinoma tumors.

Combined liver Ad untargeting with trimeric bi-specific adaptors to reduce complications of hepatic viral infection, transductional tumor retargeting to increase efficacy and specificity of transgene expression in hepatic tumors, and transcriptional restriction to enhance tumor-specific transgene expression suggests a means to engineer practical, effective therapeutic agents for hepatic CRC metastases in particular, as well as hepatic metastases of other epithelial cancers.

Keywords: adenovirus retargeting, COX-2, colorectal carcinoma

205 Radiation-Induced TNF-alpha Therapy in Prostate Cancer

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Radiotherapy (RT), in combination with androgen ablation is a standard treatment for patients with poor prognosis localized prostate cancer. Nevertheless, outcomes are suboptimal in part due to inadequate local control, which may result in eventual spread to distant sites. The addition of radiosensitizing agents would potentially increase local control at current radiation doses, or potentially allow patients to receive lower RT doses with associated lower toxicities. TNF-α is a potent radiosensitizing anti-tumor agent, but toxicity limits its use as a systemic drug. Ad.Egr-TNF.11D (TNFerade™, GenVec, Gaithersburg, MD) is a replication deficient E1, E3, E4 deleted adenoviral vector that encodes RT-inducible sequences upstream from a cDNA for human TNF-α. In pre-clinical models, RT-induced intratumoral TNF-α results in enhanced tumor regression via vascular destruction and thrombosis. Phase I and II trials of Ad.Egr-TNF.11D and RT have demonstrated safety and potential efficacy in sarcoma, esophageal, head and neck and rectal cancers, and is currently undergoing phase III evaluation in pancreatic cancer. Patients with high risk localized prostate cancer have an increased incidence of local recurrence, which may translate into distant recurrence and decreased survival making it a viable clinical venue for further clinical and translational research. Indeed, higher radiotherapy doses have led to improved disease control, but further improvements are limited by current technology, cost and normal tissue toxicity.

Based on these data, adding Ad.Egr-TNF.11D to standard radiotherapy and androgen ablation has potential, but requires formal safety evaluation. A phase I study incorporating a novel Bayesian continuous toxicity monitoring scheme (TITE-CRM) has thus been planned. Even as clinical development of Ad.Egr-TNF.11D with RT proceeds, the mechanisms mediating resistance and treatments to overcome such resistance need to be determined. Furthermore, selection of patients and tumors resistant to standard RT would help define the population for future phase II and III trials. Our data to date suggest that RT not only induces STAT1, but that STAT1 and an associated 7-gene expression profile are mechanistically important for radiation resistance, raising the hypothesis that these may be predictive biomarkers. In addition, activation of NFκB by both TNF and radiation may be critical to promoting survival and inhibiting both the cancer and endothelial cell death required for successful treatment. Further preclinical studies inhibiting NFκB activation with small molecules in the context of this treatment are in process. Ongoing studies utilizing SPORE tissue resources furthermore seek to determine the prognostic and predictive value of STAT1 and the associated 7-gene signature in patients previously treated with surgery or RT.

Keywords: gene therapy, radiotherapy, TNF-alpha

206 Double-Targeted Macromolecular Therapeutics for the Treatment of Prostate Cancer

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Binding of drugs to water-soluble polymeric carriers (polymer-drug conjugates; macromolecular therapeutics) results in numerous advantages when compared to low-molecular weight drugs: a) active uptake by fluid-phase pinocytosis (non-targeted polymer-bound drugs) or receptor-mediated endocytosis (targeted polymer-bound drugs), b) increased *active* accumulation of the drug at the tumor site by targeting, c) increased *passive* accumulation of the drug at the tumor site by the enhanced permeability and retention (EPR) effect, d) long-lasting circulation in the bloodstream, e) decreased non-specific toxicity of the conjugated drug, f) decreased immunogenicity of the targeting moiety, g) immunoprotecting and immunomobilizing activities, and h) modulation of the cell signaling and apoptotic pathways.

Clinical trials of macromolecular therapeutics demonstrated reduced side effects, increased therapeutic efficacy, and improved patient compliance [1,2]. Nevertheless, there is a need to develop a second generation of macromolecular therapeutics possessing further improved features, such as, longer intravascular half-lives, and a potential for double targeting – to the tumor cells and to a crucial subcellular organelle.

Our research is focused on nanosized (5-30 nm) water-soluble conjugates based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers. Their biocompatibility was proven in Phase I/II clinical trials in the UK. For example, doxorubicin (DOX) possesses serious cardiotoxicity; its maximum tolerated dose (MTD) in humans is 60-80 mg/m^2. The MTD of HPMA copolymer-DOX conjugate in Phase I clinical trial was 320 mg/m^2 mainly due to the fact that endocytosis is not very active in heart tissue.

The main aim of the present studies (RO1 CA132831-01) is to design, synthesize, and evaluate novel double-targeted macromolecular therapeutics containing an HPMA copolymer backbone, a targeting moiety (monoclonal antibody against prostate-specific membrane antigen (PSMA)), and a mitochondrial apoptosis inducer, e.g., (*(E)*-4-[3-(1-adamantyl)-4-hydroxyphenyl]-3-chlorocinnamic acid (3Cl-AHPC)) as a therapeutic drug. We hypothesize that this conjugate will demonstrate a dramatically improved therapeutic index in androgen-independent prostate cancer. The efficacy of targeted HPMA copolymer – 3Cl-AHPC conjugates is based on their double-targeting capacity: i) targeting to prostate cancer cells to precisely deliver therapeutic drug while reducing systemic toxicity; and ii) targeting to mitochondria by the inherent mitochondriotropism of the apoptosis inducer (3Cl-AHPC) that activates a signaling pathway leading to simultaneous engagement of the suppression of antiapoptotic effects and the activation of proapoptotic effects.

References: (1) C. Li, S. Wallace, Adv. Drug Delivery Rev. 60, 886-898 (2008); (2) H. Pan, J. Kopeček, Multifunctional Water-Soluble Polymers for Drug Delivery, In: Multifunctional Pharmaceutical Nanocarriers (Fundamental Biomedical Technologies, Vol. 4) V.P. Torchilin, Ed., Springer, New York, 2008, pp. 81-142.

Keywords: macromolecular therapeutics, prostate cancer, subcellular targeting.

207 Papovaviruses as Tools for Cancer Research

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Papovaviruses are DNA viruses with very small genomes; yet they are both transforming and tumorigenic. The human Papilloma viruses are well-established malignant tumor- generating agents in man. Polyoma virus is a murine carcinogen, and recent evidence suggests that an SV40-like agent triggers the development of an aggressive human skin cancer, Merkel tumor. These viruses each encode a very small number of proteins, of which only a fraction act to elicit a neoplastic phenotype and to sustain tumor development. Through their analysis, major insights into widely encountered molecular events that trigger and maintain a neoplastic state have emerged. As an example of the type of contribution emerging from Papovavirus research, earlier work by this Program (Tom Roberts and Brian Schaffhausen, in collaboration with Lew Cantley), led to the discovery of the role of PI3K in mammalian oncogenesis. Subsequent work, significant parts of which were conducted by this Program, identified physiologically important connections of many cancer-associated proteins to the PI3K pathway, including ras, erbB family members, the phosphatase, PTEN, TSC 1 and 2, and the protein kinases PDK1, Akt, mTOR, as well as the transcription factor, FoxO1/O3. Thus, alterations in the PI3K pathway are implicated in many human tumor types. Alterations of PI3K itself, including activating mutations, have now been detected in high prevalence in a number of common human cancers (eg, breast, colon). Because of these discoveries, PI3K inhibition is now a major goal of large pharma, with many development programs underway and several new drugs in early clinical trials. Indeed, this Program has also generated insights that have spurred current PI3K small molecule inhibitor discovery and development.

In our continuing Program, an interactive research plan aimed at gaining ever deeper mechanistic understanding of papovaviral transformation and tumorigenesis is underway. Six projects, collectively, investigate major mysteries in the field: how cells escape and how they reenter the cell cycle, both naturally and when stimulated by papovaviral T antigens; why and how two, related papovaviral small T antigens can act as an oncogene and as a potential tumor suppressor, respectively; how papovaviral small T perturbation of the protein phosphatase, PP2A, triggers powerful transforming signals through corruption of specific cellular signal transduction events; why and how SV40 large T antigen engages a mitotic checkpoint kinase as a key event in its transforming action; and how a papilloma virus regulatory protein (E2) operates as a tumor suppressing element. The goal of this Program is to continue to shed new light on cellular transformation events that also underpin human cancer development and generate insights that lead to new cancer therapeutic strategies. Among the key molecules under study are several that might ultimately prove to be useful targets for new therapeutic agents.

Keywords: PI3K, papovavirus, cancer drug targets, PI3K

208 PSMA ADC, an Auristatin-Conjugated Fully Human Monoclonal Antibody to Prostate-Specific Membrane Antigen, for Treatment of Prostate Cancer

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Prostate cancer is the most common cancer affecting men and accounts for ~30,000 deaths annually in the United States. The primary organ-confined cases can effectively be treated and cured by surgery and/or radiation therapy. Relapsed or more advanced disease can be controlled temporarily with androgen ablation. However, in virtually all patients, the tumor ultimately becomes hormone refractory. The only approved chemotherapy for hormone-refractory disease (docetaxel in combination with prednisone) provides a modest survival benefit. Therefore, there is an urgent need for novel, molecularly targeted therapies for advanced prostate cancer.

Prostate-specific membrane antigen (PSMA) is an attractive target for antibody therapy of prostate cancer due to its abundant and restricted expression on the surface of prostate cancer cells and its up-regulation in progressive and metastatic disease. We have generated a novel antibody-drug conjugate (ADC) by linking a fully human PSMA monoclonal antibody to monomethylauristatin E (MMAE), a potent inhibitor of tubulin polymerization. Here, we describe the preclinical evaluation of PSMA ADC for antitumor activity *in vitro* and in mouse xenograft models of androgen-independent human prostate cancer.

PSMA ADC eliminated PSMA-positive cells *in vitro* at picomolar concentrations and with ~1,000-fold selectivity, compared to PSMA-negative cells. The findings are consistent with the abundant expression and rapid internalization of PSMA in prostate cancer cells. In addition, PSMA ADC induced a G2/M arrest in cell cycle on PSMA-positive cell lines, consistent with the known antimitotic effects of auristatin drugs.

PSMA ADC showed therapeutic efficacy in two mouse xenograft models of androgen-independent prostate cancer. In a first study in mice (n=5/group) that were implanted intramuscularly with C4-2 cells, PSMA ADC was effective in increasing median survival and decreasing serum PSA in a dose-dependent fashion. Treatment with 6 mg/kg PSMA ADC (q4d x 6) improved survival nine-fold relative to the vehicle control group ($p = 0.0018$). At day 500, 40% (2 of 5) animals had no evidence of tumor and had no measurable PSA. In a second study in a subcutaneous C4-2 model, significant reductions in tumor size versus control were observed five days after initiation of treatment with PSMA ADC (10 mg/kg q3d x 2) ($p = 0.001$) and at all subsequent timepoints. At the end of the study (150 days post-tumor implantation), five out of six control animals had died due to tumor progression with a median survival time of 57 days. In contrast, nine of 10 animals in the PSMA ADC group were still alive at 150 days, and seven of these animals had no measurable tumor. No overt toxicity was observed in either study.

PSMA ADC has demonstrated encouraging potency and selectivity for PSMA-expressing cancer cells in *in vitro* and *in vivo* models of human prostate cancer. These results support clinical testing of PSMA ADC in men with advanced prostate cancer.

Keywords: prostate cancer, prostate-specific membrane antigen (PSMA), antibody-drug conjugate (ADC)

209 Clinical Development of 4-Hydroperoxyifosfamide (4-HOOI)

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DEKK-TEC, Inc.; CancerMedica, LLC; Southern Research Institute; Empire BioDiscovery, LLC; MPI Research; M. D. Anderson Cancer Center; Tulane Medical Center; University of New Orleans

4-HOOI is a pro-drug of isophosphoramide mustard (IPM) [the active metabolite of Ifosfamide (IFOS)] - a bi-functional DNA alkylator that generates guanine-guanine interstrand cross-linking in

G-X-C sequences producing cell death. IPM is the ultimate alkylator; however, due to polarity and high renal clearance with tubule necrosis, its optimal therapeutic doses may be precluded (AACR, 46, 4193, 2005). IFOS therapy is hampered by requiring hepatic activation and releasing extracellular acrolein and chloroacetaldehyde - resulting in dose limiting cystitis and in neurotoxicity, plus myelosuppression. 4-HOOI spontaneously undergoes ring cleavage releasing acrolein and chloroacetaldehyde primarily *in situ* in cancer cells, not extracellularly in the general circulation and has not been associated with cystitis, renal tubular necrosis and/or CNS toxicity during therapy.

4-HOOI has been screened in preclinical sub-acute pharmacology and toxicology models and in 20+ human xenograft tumor models.

Response of Human Tumor Xenografts Growing in Mice

Drug	Dose (mg/kg/day)	No of Mice	MX-1* T– C (Days)	U251** T - C (Days)	P388/CPA*** % ILS
4-HOOI	60 (<LD10)	10	28.8	84	+209^{+}
IFOS	40 (MTD)++	10	8.6	18	+42
IPM	40 (MTD)++	10	2.1	NA	+85

*Breast – SC, **glioma – IC, ***CPA (cyclophosphamide) - resistant leukemia. Dose - 5-Day Schedule (qd 1-5); for MX-1 & U251 – T - C (days) = difference in median times post implant for tumors of treated groups to attain an evaluation size compared to median of control group; $^{+}$6-log cell kill; $^{++}$>40 mg/kg was too toxic.

Our interest in 4-HOOI arises from its improved anticancer activities when compared to IPM and IFOS *vs*. the drug-resistant human MX-1 breast cancer xenograft, the U251 glioblastoma, ZR-75-1 breast cancer (with 83% surviving), as well as *vs*. CPA resistant murine leukemias. The U251 data is impressive considering BCNU produced a 72% ILS. Myelosuppression was the DLT in mice @ LD_{10} 300 mg/m^2 for a single dose and @ 180 mg/m^2 qd x 5d. No convulsions, neuropathies or renal dysfunctions were observed. Dog studies will be completed and presented. 4-HOOI did not generate any detectable plasma chloroacetaldehyde *vs*. IFOS (which generated 2.12 µg/mL from 400 mg/kg) and only 25% of the acrolein generated from the MTD of CPA and IFOS.

Unlike IFOS, 4-HOOI is more lipophilic, activated *in situ* (with less extracellularly acrolein and no chloroacetaldehyde released); no IFOS or IPM-associated CNS or GU toxicity was noted. 4-HOOI is being readied for Phase 1 trials in humans @ TMC & MDA – breast cancer, gliomas, and CPA-resistant leukemia, where it may increase the safety and efficacy margins of this class of alkylators in advanced CPA- and IFOS-resistant cancers and broaden the target-range (CNS-gliomas). Bulk and clinical product stabilization and manufacture will be presented. Supported by grants R43/44 CA094566 from the NCI/SBIR program.

Keywords: 4-HOOI, glioblastoma, breast cancer

210 Novel Radio-Viro Therapy for Prostate Cancer Using the Sodium-Iodide Symporter and 131-I: A Phase I Clinical Trial of Cancer Gene Therapy

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Radioiodine (^{131}I) is utilized routinely for therapy of recurrent and metastatic thyroid cancer because of the native expression of NIS in that tumor; our protocol seeks to bring this effective therapy to men with prostate cancer. Ad-CMV-NIS is an E1A deleted, replication deficient adenovirus, containing the cDNA for the human sodium-iodide symporter (NIS), and the cytomegalovirus early gene promoter. The product of the NIS gene is the NIS protein, that is normally expressed by functioning thyroid tissues and there causes uptake and concentration of iodine. Infection of prostate cancer cells with Ad-CMV-NIS causes expression of the NIS protein, which induces high level uptake and concentration of iodine by the prostate cells. This induced iodine uptake allows imaging and therapy of the prostate cancer by administration of radioactive iodine as we have demonstrated in preclinical small and large animal models.

We have received approval from FDA (IND# BB-12539), RAC, and local IRB and ancillary committees to open a phase I clinical trial of Ad-CMV-NIS and anticipate opening in August 2008. Viral particles will be directly injected into the tumors of men with prostate cancer that is locally recurrent following external beam radiotherapy. The virus will be injected trans-perineally thru needles inserted by trans-rectal ultrasound imaging, a method similar to that practiced for prostate brachytherapy. NIS expression, reflected by iodine uptake, will be monitored and quantitated using SPECT-CT fusion imaging following ^{123}I administration and dosimetry will be performed. A single therapeutic dose of ^{131}I, the quantity determined by dosimetry measurements (maximum dose of 200 mCi), will be administered to the subjects. Four doses will be evaluated in up to 17 men beginning at 10^9 viral particles and maximum dose will be 10^{12} particles, based upon tolerance. The patients will be followed by PSA measurements and imaging for response determination of the MTD utilizing standard endpoints.

Keywords: gene therapy, sodium-iodide symporter, radioactive iodide

211 Proteomic Mapping of Endothelial Caveolae to Pump Radio-Antibodies Into Tumors for Specific Imaging and Therapy

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Cancer and other disease biomarkers and targets may provide key diagnostic, prognostic and therapeutic opportunities including clinical trial surrogates and screens for patient treatment assignment. Drugs, gene vectors, and nanoparticles may benefit greatly from improved in vivo delivery through homing to specific disease biomarkers. Yet in vivo barriers limit access to most disease targets including cancer. We have developed novel systems biology approaches that integrate nanotechnology-based subcellular fractionation, quantitative organellar & subtractive proteomics, bioinformatic interrogation, antibody generation, expression profiling, and various in vivo imaging modalities to quickly identify and validate target candidates for pre-clinical and clinical testing. Analysis of rodent and human tumor samples have been compared to focus on clinical meaningful targets and to understand model relevance to human disease. Tissue and tumor microenvironmental influences on endothelial cell expression are extensive. We have developed quantitative proteomic analysis using a new spectral intensity index to identify proteins specific to tumor vs. normal endothelium as well as concentrated in caveolae; many of which are confirmed by immuno-electron microscopy. Novel targets in caveolae enable antibodies to penetrate deep into solid tumors and single organs and were utilized to improve tissue-specific imaging and treatment. Our recent findings reveal that caveolae not only express tissue-specific proteins but also function to rapidly and actively pump specifically targeted antibodies and nanoparticles across the endothelial cell barrier and into the tissue interstitium. This targeted penetration of the antibody into the tissue (transcytosis) occurs within seconds to minutes in normal tissues and with in minutes to a few hours in various tumor models tested. Such pervasive access inside the tumor improves the efficacy of radioimmunotherapy in destroying both stromal and tumor cells and in treating a wide variety of solid tumors. The first antibody that we wish to test clinically recognizes annexin A1 which appears tumor-induced and –specific on the outside surface of endothelia in vivo based on proteomic imaging data already published (Oh et al., Nature, 2004). Various rodent tumors are imaged rapidly and specifically after intravenous injection of specific monoclonal antibodies. This radioimmunotherapy effectively destroys tumors in rodent models to increase survival and even apparently cure the disease. So far, we have tested breast, lung, ovarian, prostate, and liver tumors with similar success. We have antibodies that recognize this target in humans. A wide variety of human tumors express this novel accessible endothelial cell surface target in a pattern quite similar to the rodent models. We are testing different radionuclides to evaluate which one is most effective. Toxicology studies are ongoing. Our antibody appears useful in tumor-specific imaging as well as in treating a wide variety of solid tumors. This work represents a novel discovery, validation and delivery strategy that so far provides promising and unprecedented results. Testing in humans is now necessary to understand limitations and possibilities for clinical translation to imaging and treating human disease.

Keywords: vascular endothelium, radiommmuno-imaging, radiommmuno-therapy

212 Taxane-Based Tumor-Targeting Anticancer Agents

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The most serious long-standing problem in cancer chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity, which leads to systemic toxicity, causing undesirable severe side effects. Therefore, various drug delivery protocols and systems have been explored in the last decades, including our laboratories. In general, a tumor-targeting drug delivery system consists of a tumor recognition moiety and a cytotoxic warhead connected directly or through a suitable linker to form a conjugate. The conjugate, which can be regarded as "guided molecular missile", should be systemically non-toxic. This means that the linker must be stable in blood circulation. Upon internalization into the cancer cell, however, the conjugate should be readily cleaved to regenerate the active cytotoxic warhead.

This presentation will report our research program on the discovery and development of new taxane-based anticancer agents possessing tumor-targeting ability and efficacy against various cancer types, especially drug-resistant tumors. These new tumor-targeting anticancer agents (TTACs) are conjugates of the 2nd-generation taxoid anticancer agents with tumortargeting modules through mechanism-based cleavable linkers. TTACs are specifically delivered to tumors, internalized into tumor cells, and the potent taxoid anticancer agents are released from the linker into the cytoplasm. We used omega-3 polyunsaturated fatty acids, in particular DHA, and monoclonal antibodies (for EGFR) as tumor-targeting molecules for drug conjugates, which exhibited excellent efficacy against human tumor xenografts (colon, ovarian, squamous) in mouse models. Vitamin receptors are excellent biomarkers for cancers. Thus, biotin and folate were successfully employed as tumor-targeting molecules as well. In order to monitor and elucidate the mechanism of tumor-targeting, internalization and drug release, several fluorescent and fluorogenic probes were developed and we succeeded in monitoring the receptor-mediated endocytosis, drug release, and drug-binding to the target protein, microtubules, by means of confocal fluorescence microscopy. The use of functionalized single-wall carbon nanotubes as a vehicle for drug conjugates bearing multiple guiding modules and warheads was also studied, which led to the development of novel nanopharmaceutical agents. Those novel drug conjugates, targeting vitamin receptors, exhibit remarkable biomarker-specificity and practically non-cytotoxic to human fibroblast cells. Prospects of these tumor-targeting anticancer agents from preclinical studies to translational research will also be presented.

Keywords: drug delivery, tumor targeting, taxane

213 Lipidic Nanoparticle-Based Drugs for Brain Tumor Treatment

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We have developed novel nanoparticle agents that can be used to treat brain tumors, either via systemic administration or via convection-enhanced delivery (CED) with MRI-guidance. For systemic treatment, nanoliposomal CPT-11 is a novel liposome-based nanoparticle featuring extremely high efficiency drug loading and stabilization. In the rat intracranial U87 tumor xenograft model, i.v. treatment with nanoliposomal CPT-11 produced 13-fold higher drug exposure in tumors based on tissue AUC than free CPT-11. Systemic treatment with nanoliposomal CPT-11 resulted in significantly improved survival, including apparent cures in some animals, as compared with free CPT-11. Based on these results, nanoliposomal CPT-11 is proceeding to Phase I clinical testing in advanced brain tumor patients.

For CED, studies in rodents and primates established the feasibility of MRI-based monitoring of CED of liposomal Gd. We further investigated the safety and efficacy of CED using liposome-based nanoparticles containing gadolinium and CPT-11 in a veterinary clinical trial in spontaneous canine gliomas. CED was monitored in real time by sequential MRI to assess localization and volume of distribution. Successful intratumoral delivery of nanoliposomal CPT-11 was demonstrated in all animals by MRI, although significant variability was observed and appeared related to catheter placement, rate of infusion, presence of necrosis and location in relation to ventricular and subarachnoid spaces. Infusions of up to 777 ul were feasible over 3.75 h using 1-3 catheters and infusion rates up to 4 ul/min. Leakage into ventricular or subarachnoid spaces was detected by MRI. Efficacy of this treatment was clearly observed, with decreases in tumor volume of up to 80% on MRI; dogs showed improvement in clinical signs following treatment. Anti-tumor effects appeared to correlate with MRI-based delineation of extent and localization of infusions. Serial exams revealed minimal evidence of neurotoxicity. Postmortem histopathologic analysis in a subset of dogs was correlated with in-life MRI data. Significant differences were seen between infused and non-infused tumor tissue, including presence of frank tumor necrosis and histologic evidence of treatment effect in infused areas. No tissue injury was observed in surrounding normal brain. These studies indicate that MRI-guided CED infusion of liposome-based agents into spontaneous canine gliomas is feasible, well-tolerated and efficacious. We conclude that this approach warrants further investigation for the treatment of human brain tumors.

Keywords: nanoparticles, convection-enhanced delivery, MRI

214 Novel Liposome and Immunoliposome Drugs Against Aggressive Breast Cancer Subtypes

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We have developed new approaches to cancer treatment involving next-generation lipidic nanoparticle agents, including mAb fragment targeted immunoliposomes (ILs) and nanoliposome drugs featuring novel drug loading and stabilization technologies. For molecular targeting, Fab' and scFv against HER2 were used to internalize liposomal drugs in HER2-overexpressing models in vitro and in vivo, resulting in superior antitumor efficacy as compared to free drug, non-targeted liposomal drug, free mAb and combinations. Anti-HER2 immunoliposome doxorubicin (NSC701315) has been GMP manufactured and licensed to industry. To target EGFR, Fab' and scFv against EGFR were similarly used to generate anti-EGFR immunoliposomes. Preclinical studies indicated superiority for this approach over other treatment conditions. Anti-EGFR immunoliposome doxorubicin has been GMP manufactured and is currently undergoing Phase I clinical testing (Univ. Basel, Switzerland). New technologies for modified gradient-based drug loading and stabilization have yielded novel "nanoliposome" drugs (nanoliposomes/nLs) for delivery of camptothecins and other cytotoxic compounds. Nanoliposomal CPT-11 has shown high drug loading efficiency (100%), extremely high drug yield (10e5 drugs/particle) prolonged circulation as an intact particle in multiple species, and significantly improved therapeutic index in various models. Nanoliposomal CPT-11 (PEP02) has completed an initial Phase I trial and is currently in Phase II testing in Asia and Europe.

We also hypothesized that lipidic nanoparticle agents can be developed against aggressive breast cancer phenotypes for which standard treatment is inadequate, especially basal-like tumors with their asssociated cancer stem cell characteristics. These poor prognosis tumors, typically hormone receptor(-) and HER2(-), lack effective targeted therapies and appear to have distinct chemotherapy response profiles. To generate antibodies capable of targeting nanoparticle drugs to subtype-specific breast cancer cells, we selected cell lines representative of luminal and basal breast cancers from a panel of 55 well-characterized breast cancer cell lines. Basal and luminal subtype-specific mAbs were identified via direct selection of human phage antibody libraries against these cell lines. Phage antibodies selected upon basal-like cell lines were confirmed to be largely basal subtype-specific, with minimal binding to luminal breast cancers. When conjugated to liposomes, mAb candidates mediated rapid binding and internalization of immunoliposomes in basal-like tumor cells. For antigen discovery, we used yeast display of tumor associated antigens to identify two basal subtype-specific phage antibodies as anti-EphA2 and anti-CD44. Anti-EphA2 antibody 2D6 competed with Ephrin A1 for cell binding and inhibited the invasion of MDAMB231 cells. Dual coupling of anti-EphA2 antibody 2D6 and anti-EGFR antibody P2/4 to liposomes resulted in enhanced liposomal uptake in basal type breast cancer, suggesting a synergistic effect between EphA2 and EGFR targeting. Anti-basaloid immunoliposome drugs may provide a novel therapeutic approach for these aggressive tumors.

Keywords: nanoparticles, immunoliposomes, phage antibodies

215 Ad-sTRAIL, an Adenoviral Construct Expressing Soluble TRAIL, Improves Survival in a Bioluminescence Imageable Intracranial Xenograft Model and an *ex-Vivo* Model of Malignant Glioma

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Malignant cells exhibit resistance to apoptosis and selective activation of pro-survival pathways that enable them to proliferate and survive in adverse conditions. However, many malignancies express receptors to Apo2L/TRAIL (TNF-α related apoptosis inducing ligand), a death inducing ligand that selectively induces apoptosis in tumor cells. Being a soluble ligand, TRAIL may potentially be a suitable agent for therapy of locoregional tumors such as malignant gliomas. We have previously demonstrated that TRAIL can robustly induce apoptosis in glioma cells and can interact with the Akt-mediated survival pathway.

In this study, we generated an adenoviral construct that expresses the soluble extracellular portion of TRAIL (Ad-sTRAIL) and tested its *in vitro* activity and in vivo activity. We also tested the *in vivo* effects of this agent against an intracranial glioma xenograft model in nude mice using U251HF and SNB19 glioma cells stably transfected with luciferase allowing bioluminescent imaging of tumor growth with intratumoral Ad-sTRAIL injections given twice weekly for 4 weeks with injection of Ad-EGFP and PBS as controls. Tumors formed by SNB19 and U251HF cells displayed characteristics of human high grade gliomas including high cellularity, pleomorphism, vascular proliferation and necrosis. Ad-sTRAIL and Ad-EGFP treated tumors showed adenoviral hexon protein expression. Tumors treated with Ad-sTRAIL, but not Ad-EGFP or PBS, showed caspase 3 activation and TUNEL positivity by immunohistochemical staining indicating selective induction of apoptosis by the agent. Bioluminescent imaging at days 7, 14, 21 and 28 demonstrated unrestricted growth of tumors in the PBS and Ad-EGFP treated animals but strong inhibition of tumor growth in the Ad-sTRAIL treated tumors. Ad-sTRAIL treated animals also showed prolonged overall survival compared with control animals suggesting that this agent has potential for clinical activity against malignant gliomas.

To further examine these effects in human tissue, we developed a human glioblastoma ex-vivo organotypic slice culture model; Upon treatment with Ad-sTRAIL, human glioma slices showed induction of apoptosis and caspase activation. In addition, we studied the effect of inhibition of XIAP, a potent inhibtor of death receptor mediated apoptosis, on TRAIL sensitivity in the U251HF intracranial glioma model. Intratumoral treatment with Ad-XAF1 (expressing XAF1 which binds and inhibits XIAP inhibitor) prior to treatment with Ads-TRAIL, resulted not only in durable tumor responses but also significantly improved survival compared with Ads-TRAIL alone. Our results suggest that additional studies of Ad-sTRAIL, in combination with strategies overcoming potential resistance mechanisms of death receptor induced apoptosis, against malignant gliomas are warranted to fully explore its potential as a therapeutic agent.

Keywords: apoptosis, TRAIL, glioma

216 Successful Oncology Translational Research: The UAB-University of Chicago Experience With Engineered HSV for Glioma Therapy Program Project Grant (PPG)

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Over the past 13 years, we have developed an effective team-science approach for translation of novel concepts for the treatment of malignant gliomas based on the use of genetically-engineered herpes simplex viruses (HSV). This paradigm has resulted in the development of pioneering approaches for the design of novel therapeutic agents due to rich cross-fertilization of ideas by specialists in molecular and clinical virology, radiation oncology, glioma biology/tumor immunology, neuro-oncology and neurosurgery. Detailed analyses of clinical data have led to group definition of directions for the generation of new laboratory studies. In turn, laboratory findings have impacted on the design of clinical trials. Three different clinical trials examining the use of engineered HSV-1 in patients with malignant glioma have now been led by the neurosurgeon in the group, and cGMP virus for the fourth trial is currently being manufactured under the NCI RAID grant mechanism.

Initial clinical trial findings indicated that $\gamma_1$34.5-deleted ($\Delta\gamma_1$34.5) HSV-1 viruses were promising therapeutic agents but to be fully effective had to (i) augment local immune responses and (ii) overcome genotypic variation-based restriction of virus replication. Based on this rationale, two approaches were undertaken. The first led to the development of a human IL12-expressing $\Delta\gamma_1$34.5 HSV, M032, that is significantly more effective in preclinical studies. This virus is being produced by the RAID Program for clinical studies.

The solution to the second objective, to overcome tumor genotype-dependent restriction, is based on the observation that gliomas resistant to therapeutic viruses express an activated protein kinase R (PKR) and that the activated PKR can be effectively blocked by expressing a constitutively activated MEK. Extensive in vitro and in vivo preclinical studies showed that $\Delta\gamma_1$34.5 HSV plus 5Gy radiation (6-24 hours later) enhanced oncolysis and efficacy in experimental brain tumor animal models without compromising safety. The safety and efficacy of this approach has now been confirmed by a recently completed Phase I trial of oncolytic HSV followed by 5Gy IMRT irradiation to the tumor bed. The next generation of therapeutic viruses currently in preclinical studies include viruses that block PKR by induced expression of constitutively-activated MEK while maintaining a safety profile similar to that of the parent $\Delta\gamma_1$34.5 HSV-1.

Mutant viruses that incorporate both of these strategies are being constructed to evaluate the potential anti-glioma efficacy and safety of this combined approach.

Keywords: oncolytic, herpes simplex virus, brain tumors

217 Astatine-211 Conditioning for Nonmyeloablative Hematopoietic Stem Cell Allografts

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Allogeneic hematopoietic cell transplantation (HCT) is an important treatment modality for patients with both malignant and nonmalignant hematologic disorders. However, the application of this treatment has been limited to relatively young patients by complications related to the toxicity of the conditioning regimens used. To decrease toxicities, nonmyeloablative regimens have been developed. While these have been quite successful in major histocompatibility antigen complex (MHC)-identical transplants, where 2 Gy total body irradiation (TBI) is adequate for engraftment, in the more complex MHC-haploidentical setting much higher and toxic TBI doses are required to ensure engraftment. We currently are investigating a systemically targeted form of radiation to replace TBI, in both MHC-identical and MHC-haploidentical HCT, which would provide a treatment option for patients without MHC-matched donors. We previously investigated the use of Bismuth-213 (^{213}Bi)-labeled anti-CD45 monoclonal antibody (MAb) as a replacement for TBI in a nonmyeloablative conditioning regimen for HCT in a canine model. While this treatment was effective in allowing engraftment of marrow, the limited availability and cost associated with ^{213}Bi led to a preliminary investigation of the use of Astatine-211 (^{211}At) for the same application. Specifically, the current research efforts will determine if the alpha-emitting radionuclide ^{211}At, when conjugated to a panhematopoietic anti-CD45 MAb, can replace TBI to condition recipients for allogeneic HCT. We will utilize our well-established preclinical model of randombred dogs, which has been predictive of allogeneic HCT in humans.

In *specific aim 1,* we evaluated and optimized a new method for labeling MAbs with ^{211}At. We determined that the method, involving conjugation of a molecule containing an astatine-reactive borate(2-) moiety to the MAb followed by astatination, gave higher and more consistent labeling yields. In *specific aim 2,* studies were conducted to gain an understanding of the differences between biodistribution and myelosuppression/toxicity of ^{213}Bi- and ^{211}At-labeled rat anti-murine CD45 MAb, 30F11, in mice. The data indicate that ^{211}At is at least as effective as ^{213}Bi in myelosuppression with similar toxicity. The data is also suggestive that considerably lower quantities of ^{211}At-labeled anti-CD45 MAb than observed for ^{213}Bi-labeled MAb may be effective in myelosuppression. In *specific aim 3*, evaluations of ^{211}At-labeled MAbs will be conducted in dogs to find effective doses for both dog leukocyte antigen (DLA)-identical and DLA-haploidentical HCT. Currently, we are conducting dose-finding studies in the dog to determine the minimal dose that is effective for myelosuppression. Following that, the quantity of ^{211}At-labeled anti-CD45 MAb required to obtain stable engraftment in HCT involving DLA-identical littermates will be determined. The final, and most important, studies will involve determining the quantity of ^{211}At-labeled MAb required to obtain stable engraftment in HCT involving DLA-haploidentical littermates.

Keywords: hematopoietic cell transplantation, MHC-haploidentical, alpha-emitters

218 mda-7/IL-24: Broad-Spectrum Gene Therapeutic for Localized and Disseminated Cancers

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It is well established that cancer cells frequently display abnormalities in normal programs of differentiation, a process that is reversible in particular cancers by appropriate treatment. Our research has focused on exploiting this property of tumor cells to develop ways of pharmacologically reversing the cancer phenotype by inducing terminal differentiation, a process termed *differentiation therapy of cancer*. Exposure of human melanoma cells to fibroblast interferon and mezerein results in reversion of cancer cells to a more normal state, irreversible growth arrest and terminal cell differentiation. Using this model system combined with subtraction hybridization we cloned a number of initially novel genes involved in cell cycle control, cell growth, differentiation and apoptosis. One unique gene, melanoma differentiation associated gene-7 (*mda*-7), a new member of the interleukin (IL)-10 gene family, designated IL-24, has come to the limelight as a potentially significant gene for the therapy of multiple human cancers. *mda*-7/IL-24 displays several exceptional properties that contribute to its potential as an effective cancer gene therapy. This gene selectively induces growth suppression and apoptosis in cancer cells of diverse origin, does not harm normal cells, inhibits tumor development and progression *in vivo* in human tumor xenograft models, induces a potent antitumor 'bystander' effect, inhibits tumor angiogenesis, enhances the antitumor effects of radiation, chemotherapy and monoclonal antibody therapy and modulates immune functions. Recent studies are providing important insights into the mechanism of action of *mda*-7/IL-24 indicating selective induction of endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) in tumor cells as factors involved in cancer-specific apoptosis. In the *in vivo* setting it is likely that the combined actions of this intriguing cytokine contributes to its profound anti-cancer activity. Of significant import, *mda*-7/IL-24 has entered the clinic recapitulating many of the effects apparent in cell culture and in animal models, namely induction of tumor cell specific apoptosis, 'bystander' antitumor activity and immune modulation. In patients with advanced carcinomas or melanomas in a Phase I Clinical Trial, multiple intratumoral injections with a replication incompetent adenovirus expressing mda-7/IL-24, Ad.mda-7 (INGN 241), was shown to be safe and display significant clinical activity. Future strategies employing conditionally replicating and tropism modified adenoviruses to deliver *mda*-7/IL-24 and combining this therapy with additional therapeutic agents, such as radiation, chemotherapy and/or monoclonal antibodies, offer promise for further enhancing the therapeutic benefit of this novel cytokine.

The long-term objectives of our research programs are to develop an improved understanding and define enhanced translational applications for *mda*-7/IL-24. Based on early success in clinical trials, our studies are focusing on advancing this gene therapy into the clinic as a frontline therapy for diverse cancers. The specific aims of our PPG are to expand on the provocative basic science studies and the early clinical results of *mda*-7/IL-24 to accelerate its translational applications in three cancer indications, prostate cancer, glioblastoma multiforme and ovarian cancer. Specific projects focus on defining the regions of MDA-7/IL-24 controlling activity; mechanistically defining combinatorial action of MDA-7/IL-24 with radiation; and developing improved approaches for delivering MDA-7/IL-24 as a therapy for cancer.

Keywords: differentiation therapy of cancer, cancer-specific apoptosis, cancer gene therapy

219 Systemic Orthotopic, Anti-Pancreatic Cancer Effects *in Vivo* With a Novel Bispecific Ligand-Directed Toxin Delivered Using a New Method of Drug Delivery

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A novel recombinant bispecific ligand-directed toxin (BLT) was synthesized that had far greater activity in vitro against human pancreatic tumor lines than its monospecific counterparts fulfilling our criteria of a successful BLT. This molecule, DTEGF13, consisting of human EGF and IL-13 spliced to truncated diphtheria toxin also had greater activity than a mixture of the monospecific agents indicating an advantage of combining the two ligands on the same single chain molecule. Imaging of mice given orthotopic injection of a highly aggressive MiaPaCa-2 tumor transfected with dual luciferase/GMP reporter genes showed induction of pancreatic cancer that was metastatic to the liver. Since one of the major problems with biologicals is drug delivery, we developed a new drug delivery method called profectitious drug delivery (PDD) in which a dose of recombinant EGF13 devoid of toxin is given ip 5-10 minutes prior to the therapeutic dose of ip DTEGF13. One of the major benefits of using this PDD method is that we were able to safely exceed the MTD of DTEGF13 by about 15-fold. Multiple courses of PDD begun on day 3 resulted in the slow, but continuous regression of MiaPaCa-2 tumors in the pancreas as measured in real time using bioluminescent imaging. Several of the tumors were eliminated and did not reoccur. Similar tumor regressions were observed upon bioluminescent imaging in a second highly aggressive orthotopic pancreatic cancer model, SW1990. We believe that PDD works because the predose of EGF13 blocks receptors on normal cells but is not sufficient to block the higher number of EGF and IL13 receptors over-expressed on pancreatic cancer cells. BLTs represent a new class of biologicals that may allow us to increase the anti-tumor potency while reducing toxicity. Pharmacokinetic and toxicity data is being generated to support an IND application.

Keywords: pancreatic cancer, recombinant biological drug, animal model

220 Development of Targeted Cancer Gene Therapy

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Pancreatic cancer is an aggressive malignancy with morbidity rates almost equal to mortality rates because of the current lack of effective treatment options. Under the support of SPORE in Pancreatic Cancer, we have successfully developed a targeted gene therapy approach to treat pancreatic cancer with effective therapeutic efficacy and safety in noninvasive imaging models. We have linked VISA (VP16-GAL4-WPRE integrated systemic amplifier) with a CCKAR (cholecystokinin type A receptor) gene-based, pancreatic-cancer-specific promoter. The newly established expression vector, namely, C-VISA (CCKAR-VISA) was shown to target transgene expression in pancreatic tumors in vivo. Targeted expression of BikDD, a potent proapoptotic gene-driven by C-VISA, exhibited significant antitumor effects on pancreatic cancer and prolonged survival in multiple xenograft and syngeneic orthotopic mouse models of pancreatic tumors with virtually no toxicity (Cancer Cell 12:52-65, 2007).

We are in the process of conducting safety studies to initiate an IND (Investigational New Drug Application) and move CCKAR-VISA-BikDD into a Phase I clinical trial for pancreatic cancer. A protocol entitled "A Phase I Open-Label Dose Escalation Study to Assess the Safety and Tolerability of the BikDD Nanoparticle in Patients with Advanced Pancreatic Cancer" has been submitted and reviewed by the NIH Recombinant DNA Advisory Committee (RAC) on June 17, 2008, which provided some minor recommendations to improve the protocol.

Keywords: VISA, BikDD, CCKAR

221 Developing Combination Therapies for Hormone-Refractory Prostate Cancer in a Preclinical Mouse Model of the Disease

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Although most men diagnosed with early stage prostate cancer have favorable outcomes, those with advanced disease and particularly hormone-refractory prostate cancer eventually succumb to lethality since treatment options are limited. We have been investigating targeted therapies for the treatment of advanced prostate cancer using a relevant genetically-engineered mouse model of the disease, namely the *Nkx3.1; Pten* mutant mice. Based on previous studies showing that the Akt/mTOR and Erk Map kinase signaling pathways cooperate in prostate cancer progression, we have now performed pre-clinical studies in the *Nkx3.1; Pten* mutant mice to examine the consequences of combinatorial inhibition of these signaling pathways for prostate tumorigenesis in androgen-dependent and -independent contexts. We report that combination therapy using Rapamycin, an inhibitor of mTOR, and PD0325901, a MEK inhibitor, is potently anti-tumorigenic in the *Nkx3.1; Pten* mutant mice, particularly in contexts of limiting androgens. Furthermore, we find that these signaling pathways are coordinately de-regulated during prostate cancer progression in humans. Based on these pre-clinical studies in the mutant mice and the supporting data from human prostate cancer, we propose that combination therapy targeting the Akt/mTOR kinase and Erk Map kinase signaling pathways may be effective for treatment of patients with advanced prostate cancer, particularly in conjunction with androgen deprivation therapy.

Keywords: Akt/mTOR signaling, hormone-refractory prostate cancer, preclinical in vivo models

222 Gene Mediated Cytotoxic Immunotherapy for Newly Diagnosed Prostate Cancer

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Advantagene, Inc.

Rationale: Current therapies for prostate cancer (PCa) provide an excellent 5yr survival prognosis for the approximately 200,000 new annual diagnoses. However, each year there are 60,000-100,000 recurrences and about 30,000 deaths. PCa recurrence, treated by surgical or pharmacologic castration, is a growing problem in the US. The magnitude of the problem may be better appreciated in the number of American men living with PCa, estimated at 2.1 million in 2005. The costs to society from treatment costs, lost productivity and compromised quality of life are in the billions of dollars. Drugs that decrease recurrence of PCa and do not diminish current success from standard therapies would be of great significance. However, PCa is a prolonged disease; efficient drug development will require identification and validation of shorter-term clinical endpoints.

Background: ProstAtak™ (Advantagene, Inc) is an adenoviral vector containing the Herpes virus thymidine-kinase gene (AdV-tk) delivered directly to the prostate followed by an antiherpetic prodrug. It kills tumor cells via necrosis and apoptosis, elicits danger signals, and stimulates anti-tumor T-cell proliferation. Preclinical data demonstrated synergy with standard surgery and radiation. Phase 1 clinical studies in multiple tumor sites, with over 300 patient doses delivered, have shown an excellent safety profile. ProstAtak™, as a monotherapy, showed objective clinical responses in recurrent prostate cancer and immune activation as a neoadjuvant to surgery. A 71 patient Phase 2a, open label, single institution study in newly diagnosed PCa in combination with radiation therapy enrolled 33 “low risk” patients (Arm A, PSA<10, Gleason<7, and T1c-T2a), that received two treatments with AdV-tk + valacyclovir, immediately before and 14 days into radiation; 33 intermediate and high-risk patients (Arm B, PSA≥10, Gleason≥7, or T2b-T3), and 5 stage D1 (Arm C, positive lymph nodes) both of the latter received an additional treatment at initiation of androgen deprivation therapy. ProstAtak™ was delivered in an outpatient setting and it was not necessary to modify any standard therapy.

Results: No significant toxicity was observed and feasibility was demonstrated. Four subjects withdrew due to non-compliance or valacyclovir intolerance. Two surrogate and one definitive efficacy end-points were evaluated. First, the frequency of patients in Arm A with PSA nadir ≤0.2 ng/ml was 77% vs 58% in a matched group of concurrent patients. Second, a two-year pathologic complete response by sextant biopsy was 91% in Arm A and 94% in Arm B, compared to 70-73% from contemporaneous large cohort studies. These surrogate end point results correlated well with the definitive freedom from failure (FFF). FFF after 68-month median follow up is 100% for Arm A and 90% for Arm B (95% for intermediate, 75% for high risk) vs the best contemporaneous large cohort results of 79-90% for low risk and 48-79% for intermediate-high risk patients. The three failures in Arm B occurred within months after treatment leading to a Kaplan-Meier curve that plateaus at 90% beyond year 3. This is notably different than previous reports in which the curves continue to drop beyond year 5.

Conclusions: The results suggest ProstAtak™ may significantly reduce recurrence in PCa patients. If confirmed, ProstAtak™ would positively impact tens of thousands of PCa patients and provide billions in economic benefit to society. A prospective, randomized controlled trial will be used to confirm the Phase 2a results and to prospectively evaluate the validity of the short-term surrogate end-points. The trial will enroll approximately 300 patients with a two-year evaluation time point for rolling into a pivotal 600 patient study.

Keywords: prostate cancer, cancer immunotherapy, AdV-tk

223 Suppression of Prostate Cancer Nodal and Systemic Metastasis by Blockade of the Lymphangiogenic Axis

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Lymph node involvement denotes a poor outcome for patients with prostate cancer. Our group, along with others, has shown that initial tumor cell dissemination to regional lymph nodes via lymphatics also promotes systemic metastasis in mouse models. The aim of this study was to investigate the efficacy of suppressive therapies targeting either the angiogenic or lymphangiogenic axis in inhibiting regional lymph node and systemic metastasis in subcutaneous and orthotopic prostate tumor xenografts. Both androgen-dependent and more aggressive, androgen-independent prostate tumors were employed in our investigations. Interestingly, we observed that the threshold for dissemination is lower in the vascular-rich prostatic microenvironment compared to subcutaneously grafted tumors. Both VEGF-C ligand trap (sVEGFR-3) and antibody directed against VEGFR-3 (mF4-31C1) significantly reduced tumor lymphangiogenesis and metastasis to regional lymph node and distal vital organs, without influencing tumor growth. Conversely, angiogenic blockade by short-hairpin RNA against VEGF or anti-VEGFR-2 antibody (DC101) reduced tumor blood vessel density, significantly delayed tumor growth, and reduced systemic metastasis, although was ineffective in reducing lymphangiogenesis or nodal metastasis. Collectively, these data clarify the utility of vascular therapeutics in prostate tumor growth and metastasis, particularly in the context of the prostate microenvironment. Our findings highlight the importance of lymphangiogenic therapies in the control of regional lymph node and systemic metastasis.

Keywords: lymph node, lymphangiogenesis, metastasis

224 The Role of the PI3K/PTEN Signaling Network in Prostate Cancer

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Hyperactivation of the phosphoinositide 3-kinase (PI3K) signaling pathway due to loss-of-function mutations in the negative regulator, PTEN occurs frequently in aggressive forms of prostate cancer. Our P01-supported research focuses on understanding the components of this pathway involved in cell growth and cell survival and on generating mouse models that interrogate the role that these components play in prostate cancer. Research supported by this P01 as well as research from other laboratories has shown that deletion of alleles of PTEN or introduction of activated forms of PI3K or AKT into the mouse prostate results in varying degrees of neoplasia from prostate intraepithelial neoplasia (PIN) to invasive carcinomas. We have also used mouse models to address the role of components of the PI3K signaling network in driving tumor formation. In particular we have found that activation of PI3K and AKT results in activation of the mTOR/p70-S6-Kinase pathway, ultimately leading to increased expression of HIF-dependent genes in the prostate. These results predicted that glucose uptake and metabolism should be enhanced in prostate cancers that have activation of the PI3K pathway and that these tumors should be positive for FDG-PET imaging. More importantly, these studies suggest that quantitative reduction of FDG-PET could be an endpoint for identifying patients who will respond to drugs that target this pathway. Finally, we have found that multiple isoforms of PI3K p110 subunits contribute to the growth of PTEN-/- prostate tumors, suggesting that drugs that broadly target class Ia PI3K isoforms may be more potent in treating prostate cancers.

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Keywords: PI3K, PTEN, AKT

225 Molecular Effects of Nutritional Supplements on the Prostate Microenvironment

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(Drs. Haqq and Carroll share senior authorship on this study)

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Background: Observational epidemiologic and laboratory data have indicated that tomatoes (rich in the antioxidant lycopene) and fish oil (rich in omega-3 fatty acids) may deter prostate cancer incidence. There has been extremely limited data focused on the possible roles of these nutrients after diagnosis. Examining changes in the prostate tissue from men on active surveillance regimens provides a unique opportunity to study the natural progression of prostate cancer *in vivo*.

Design and Methods: We conducted a randomized blinded placebo-controlled clinical trial of tomato extract and fish oil in men with low-burden prostate cancer, who elected active surveillance as their primary management strategy. Men received a placebo, 3g of fish oil supplement, or a tomato extract supplement containing 30 mg lycopene for 3 months. Our main outcome was change in expression in genes of interest (e.g. in the *IGF-I* and *COX2* pathways) based on biopsies taken pre- and post-intervention, using cDNA expression array analyses.

Results: Ninety-five men were randomized in this study, but 11 became ineligible due to non-compliance, disease progression, or voluntary withdrawal. 84 participants who completed the intervention were eligible for final analyses. Analysis of the baseline data indicates the similarity in gene expression profiles across treatment arms. We remain blinded and statistical analyses are ongoing for our primary outcomes.

Conclusions: At this stage, this trial indicates the feasibility of enrolling and conducting translational nutritional intervention research in men electing active surveillance for prostate cancer. Analyses of our primary aims are anticipated to be ready by the end of summer 2008.

Keywords: prostate cancer, fish oil, tomato

226 Early Phase Clinical Development of Polyphenon E for Cancer Prevention

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Green tea has been shown to exhibit cancer preventive activities in preclinical studies. Its consumption has been associated with decreased risk of certain types of cancers in humans. The principal active constituents in green tea are believed to be green tea catechins. Of the green tea catechins, epigallocatechin gallate (EGCG) is the most abundant and has been shown to possess potent biological activities. Our initial clinical studies examined the safety and pharmacokinetics of pure EGCG extract and a defined green tea catechin extract (Polyphenon E). Polyphenon E contains 80-98% total catechins with EGCG as the main component accounting for 50-75% of the material. The oral bioavailability of EGCG is similar between the pure EGCG product and Polyphenon E at equivalent EGCG dose levels. Both products are well tolerated in healthy individuals following chronic administration at a daily dose that contains 800 mg EGCG. Polyphenon E was selected for further clinical development because it would be less costly for long term usage in the setting of cancer prevention and it contains other catechins which also possess cancer preventive activities.

Our follow up clinical studies showed that the bioavailability of EGCG was significantly increased after repeated green tea catechin dosing at high daily bolus doses, possibly due to inhibition of presystemic elimination of this catechin. In addition, we found that taking Polyphenon E on an empty stomach resulted in a greater than 3-fold increase in the systemic bioavailability of EGCG. We also demonstrated that chronic Polyphenon E administration has minimum effect on four major cytochrome P450 isozymes, suggesting that Polyphenon E administration is not likely to affect the pharmacokinetics of commonly used medications. Chronic Polyphenon E administration was shown to induce glutathione *S*-transferase (GST) activity and GST-pi level in blood lymphocytes with the most significant change observed in individuals with low baseline enzyme activity. Our data suggest that Polyphenon E intervention may enhance the detoxification of carcinogens in individuals with low detoxification capacity. We are currently conducting multiple early phase trials of Polyphenon E to evaluate its potential for prostate, lung, and cervical cancer prevention in high risk individuals.

Supported by N01CN85719, N01CN25119, N01CN35158 from the National Cancer Institute, Division of Cancer Prevention

Keywords: green tea, Polyphenon E, cancer prevention

227 A Novel Targeted Prostate-Specific Membrane Antigen (PSMA) Activated Thapsigargin Prodrug Produces Substantial Regression and Prolonged Growth Inhibition of Human Cancer Xenografts

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Thapsigargin (TG) is a highly lipophilic, cytotoxic natural product isolated in high yield from the umbelliferous plant *Thapsia garganica*. In the NCI 60 Cancer Cell Line Screen, TG has a GI50 of ~10^{-10} M which compares favorably with chemotherapeutic agents such as Paclitaxel (10^{-8} M) and Doxorubicin (10^{-7} M) in this assay. TG is a non-cell type specific cytotoxin that kills cells in a proliferation independent manner via its potent inhibition of a critical intracellular protein, the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump. Sustained inhibition of the SERCA pump by TG produces an elevation of intracellular calcium to micromolar levels which triggers the ER stress response, releases apoptotic factors from the mitochondria and activates endonucleases within the nucleus. TG has no therapeutic index in vivo with an LD_{100} in mice of 0.2 mg/kg.

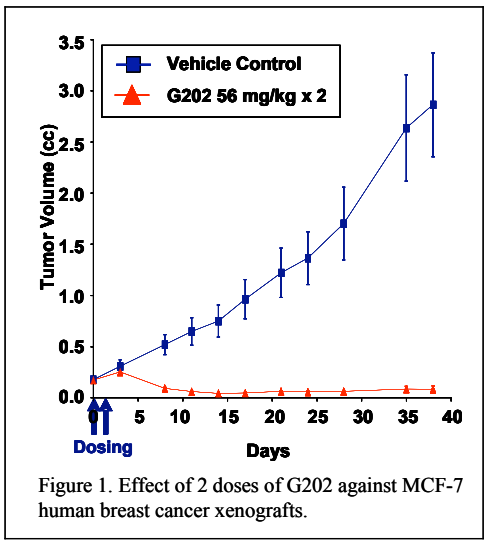

Figure 1. Effect of 2 doses of G202 against MCF-7 human breast cancer xenografts.

In order to create a therapeutic index that would allow for sytemic administration we have generated prodrugs by coupling water soluble peptides to a potent analog of thapsigargin, (12Aminododecanoyl)-8-O debutanoyl-thapsigargin (12ADT). In addition to solubilization, the peptide also serves to mask the cytotoxicity of the TG analog until it is released by specific active proteases present within tumor sites. In this study we coupled a series of acidic amino acids to 12ADT to generate water soluble prodrugs. One prodrug (G202) was selected for further in vivo evaluation based on ability to be hydrolyzed by the carboxypeptidase Prostate-Specific Membrane Antigen (PSMA). PSMA is expressed by prostate tissue with strongest expression in both primary and metastatic prostate cancers. PSMA, however, is also expressed by the neovasculature within most solid tumors including breast cancers, but not by normal tissue vasculature.

In initial pharmacokinetic studies a single dose of G202 at a dose of 56 mg/kg, IV, (i.e. ~150-fold higher TG equivalents) produced a Cmax of ~ 800 μM and a plasma half-life of ~5hr. G202 was stable to hydrolysis in blood with < 0.5% conversion observed over 24 hrs. Efficacy studies against PSMA-producing human prostate cancer xenografts in castrated mice demonstrated tumor regression and significant growth delay. G202 was also effective against human breast, bladder and renal cancer xenografts. The prodrug was particularly effective against MCF-7 breast cancer xenografts in which complete tumor regressions were observed in the majority of treated animals following a single 2 dose course of G202, figure 1. Biodistribution studies confirmed accumulation of the cleavage product Asp-12ADT in tumor tissues to micromolar levels. Levels of the Asp-12ADT were 5-100 fold lower in other normal tissues sampled. These preclinical results support the continued development and clinical testing of G202 as a novel anticancer agent for the treatment of advanced cancer.

Keywords: PSMA, prodrug, thapsigargin

228 Tomatoes, Tomato Carotenoids, and Prostate Cancer

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Prostate cancer is the most common malignancy in American men and dietary approaches that reduce its risk or delay its progression could have profound impact on public health. Epidemiological and animal studies suggest that consumption of tomato products reduces the risk of prostate cancer. Our research team from the University of Illinois and The Ohio State University has collaborated on a number of NIH-funded projects on this topic including an initial RO1 grant in 1997, a recent RO3 (Producing 14C-Phytoene & Phytofluene for Cancer Research; 1RO3CA112649-A) and a current RO1 grant awarded in 2007 (Tomatoes, Lycopene, and Prostate Carcinogenesis in Mice; 1RO1CA125384-01A1) that have focused upon the impact of bioactive components from the tomato on prostate carcinogenesis. We have utilized cell culture and various animal models and determined that lycopene, the primary tomato carotenoid, is but one of many phytochemicals found in tomatoes that may provide protection from prostate carcinogenesis. Moreover, we also hypothesize that the metabolic products of lycopene and other tomato carotenoids, not the parent molecules, may have biological activity. In our current studies we utilize several murine models to determine if the tomato carotenoids, lycopene, phytoene, and phytofluene, or their metabolic products produced by carotenoid cleavage enzymes, alter the risk of development and progression of prostate cancer. We are utilizing two novel mouse strains that lack one of the two known mammalian carotenoid cleavage enzymes, 15, 15' monooxygenase knock-out (CMO-I KO) and 9', 10' monooxygenase knock-out (CMO-II KO). The current grant addresses three major specific aims. *Specific Aim 1* is to evaluate the tissue-specific expression of CMO-I and CMO-II in A) wild-type, CMO-I KO, and CMO-II KO mice following short and long-term feeding of different levels of tomato powder or lycopene. We will then examine the expression of CMO-1 and CMO-II during prostate carcinogenesis in TRAMP mice. *Specific Aim 2* will precisely determine how changes in CMO-I and CMO-II expression dictate the tissue biodistribution of tomato carotenoids and production of lycopenoids and other tomato carotenoid metabolites. These studies will employ wild-type, CMO-I KO, and CMO-II KO mice. *Specific Aim 3* will evaluate the effect of altered tomato carotenoid metabolism on prostate cancer development by quantitating the ability of dietary tomato powder or lycopene to inhibit prostate cancinogenesis in TRAMP, TRAMP x CMO-I KO, and TRAMP x CMO-II KO mice. Our ongoing studies suggest that lycopene is metabolized primarily by the CMO-II enzyme and not cleaved by CMO-I, which is known to cleave beta carotene. Moreover, we have identified two apo-lycopenal cleavage products in rodent tissues that are of keen metabolic interest. Together, these studies will allow us to determine if tomato carotenoids, or their metabolic products, are able to modulate prostate carcinogenesis and if CMO-I/CMO-II expression defines the response to tomato carotenoids. Our preclincial studies are relevent to human application in several directions. For example, we hypothesize that genetic polymorphisms involved in the metabolism of carotenoids, such as in the genes for CMO-I and II, are critical determinants of the benefits of tomato products during prostate carcinogenesis. Our team of collaborators are uniquely qualified to carry out the proposed studies due to our broad expertise with carotenoids, experience with experimental models of prostate carcinogenesis, access to CMO-I and II KO mice, ability to biosynthesize radiolabeled tomato carotenoids using tomato cell suspension culture, and expertise in translating these findings into human clinical trials.

Keywords: lycopene, prostate cancer, carotenoids

229 Targeting the Multi-functional Cytoprotective Chaperone Hsp27 in Castrate Resistant Prostate Cancer using OGX-427: Preclinical and Phase I Clinical Observations

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Hsp27 is a multi-functional, stress-induced cytoprotective chaperone that is implicated in treatment resistance and over-expressed in many human cancers. Therapeutic targeting of Hsp27 is attractive as it would affect multiple pathways implicated in cancer progression and resistance. We previously reported that Hsp27 is highly expressed in castrate resistant prostate cancer (CRPC) and induces treatment resistance when over-expressed. Hsp27 knockdown using antisense oligonucleotides (ASO) or siRNA induced apoptosis and inhibited cell growth *in vitro* and *in* vivo in preclinical models of prostate, breast, lung, and bladder cancer. CRPC often correlates with increased activity of androgen receptor (AR), IL-6/p38 and IGF-1/ MAPK pathways and our recent studies link Hsp27 as a central 'Hub" in each of these pathways. For example, ligand-activated AR induces rapid Hsp27 phosphorylation in an AR- and p38 kinase-dependent manner, which then displaces Hsp90 to chaperone AR into the nucleus and enhance AR transcriptional activity and cell survival. Hsp27 knockdown using the antisense drug OGX-427 increases LNCaP cell apoptotic rates via proteasome-mediated AR degradation. IGF-1 also induces Hsp27 phosphorylation via MAPK pathway, specifically via a downstream effector of Erk, p90Rsk, which directly interacts with and phosphorylates Hsp27. Hsp27 inhibition leads to decreased Erk, p90Rsk and Akt phosphorylation, and destabilizes Bad/14-3-3 complexes to increase apoptotic rates. These data define interactions between phospho-activated Hsp27, AR transactivation, and the IGF-1 signalosome in CRPC progression.

Since Hsp27 modulates many varied and distinct survival pathways and networks regulating cell stress response and survival, it reveals vulnerability in cancer cells made dependent upon this chaperone to positively regulate the apoptotic rheostat. OGX-427 is a second generation 2'-methoxy-ethyl (MOE) phosphorothioate ASO which inhibits expression of Hsp27 and demonstrated preclinical activity in prostate, bladder, breast, and lung cancer models. OGX-427 is currently completing a single agent dose escalation Phase I trial in these cancers as part of the Pacific Northwest Prostate SPORE and is expected to complete by 4th quarter of 2008. The primary objectives of this study are to define the pk and toxicity profile of OGX-427 given as a 2-hour IV infusion every week and determine the recommended phase II dose (RP2D). Secondary endpoints will evaluate post-treatment PSA declines, measurable disease response and time to disease progression. Six patients per cohort were recruited at a starting dose of 200 mg IV weekly after an initial 3 loading doses given in the first week. The third dose level has just completed accrual (600 mg) with no dose limiting toxicities and patients are now accruing to the 800 mg dose level. In addition to pk and safety data, correlative studies using serial samples of circulating tumour cells enumerated as an indicator of anti-tumour activity, as well as Hsp27 expression using immunofluorescence, are being assessed. Results of this phase I translation trial will be reported.

Keywords: Hsp27, targeted therapy, chaperone

230 Tea Polyphenols in Chemoprevention of Prostate Cancer

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The anticarcinogenic potential of green (GT) and black tea (BT) has been demonstrated in many animal and *in vitro* cell culture studies. The major tea polyphenols of green tea are (-)-epigallocatechin gallate (EGCG) and (-)-epigallocatechin (EGC), whereas black tea contains smaller amounts of these polyphenols but contains larger polymeric flavonoids such as theaflavins and thearubigins. It has been demonstrated that GT and BT polyphenols inhibit cell growth through a variety of mechanisms such as antioxidant activity, alteration of redox-sensitive signal transduction pathways (nuclear factor kappa B, activator protein 1, mitogen-activated protein kinase) and inhibition of insulin-like growth factor (IGF-1) leading to the inhibition of proliferation, induction of apoptosis, cell cycle arrest as well as inhibition of angiogenesis. However most cell culture and animal studies have been performed with higher concentrations than achievable in humans. It is not clear whether effects observed in animal and cell culture studies can be applied to human studies. Therefore we are performing a phase II clinical intervention trial to investigate whether the consumption of 6 cups of GT or BT for 3-6 weeks prior to prostatectomy will decrease oxidative stress, alter signaling pathways leading to an inhibition of proliferation and increase of apoptosis in the prostate. Since in vivo polyphenols and theaflavins are subject to extensive endogenous and colonic metabolism we propose that metabolites contribute to the chemopreventive effect of GT and BT. Currently 23 participants have been enrolled. Using high performance liquid chromatography (HPLC) with coularray electrochemical detection as well as mass spectrometry (MS) EGC, EC and 4'-MeEGC were found in urine after GT consumption (0.8-2.3 μg/mL) and BT consumption (11-31 ng/mL). The majority was in glucuronidated form. After GT consumption EGC, EC, 4'-MeEGC, EGCG, 4'-MeEGCG and ECG were found in serum. EGC, EC and 4'-MeEGC were conjugated as glucuronide and small amount of sulfate, whereas EGCG, 4'-MeEGCG and ECG were mainly in the free form. Following GT and BT consumption urinary hippuric acid was increased. No theaflavins were found in urine or serum. EGCG was found in prostate tissue. No tea polyphenols were found in control participants. An interim analysis of immunohistochemical data on oxidative DNA damage, cellular proliferation, and apoptosis will be evaluated after 60 participants are enrolled. In vitro studies comparing the stability of methylated metabolite to parent compound demonstrated that methyl-EGC was stable at pH 7. Cell culture studies are being conducted to compare the effect on proliferation, apoptosis and NFκB DNA binding of metabolites to parent compounds. This phase II clinical trial will assist in the translation of *in vitro* and animal research to human application.

Keywords: green and black tea, prostate cancer, Phase II intervention trial

231 Development of Penthamethylchromanol for Prostate Cancer Prevention

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Background: About one-third of the patients with recurrent prostate cancer (CaP) drop out of standard-of-care (SOC) Androgen-Deprivation-Therapy (ADT) because of unacceptable side-effects and limited clinical benefit. In addition, current chemotherapeutic drugs have negligible success in the treatment of ADT-refractory recurrent CaP (HRPC). Direct evidence linking oxidative stress with an increase in prostate tumor development both in human and in transgenic adenocarcinoma of mouse prostate (TRAMP) model developing spontaneous prostate tumor has been reported. It has also been demonstrated that reactive oxygen species (ROS) is a key component in inducing expression of androgen-independent survival pathway in androgen-dependent cells. We have previously demonstrated that androgen treatment increases reactive oxygen species (ROS) levels in androgen-dependent CaP cells. We observed that the anti-oxidant moiety of Vitamin E 2,2,5,7,8-pentamethyl-6-chromanol (PMCol) has strong anti-androgenic and anti-inflammatory properties and is active against both androgen-dependent as well as androgen-independent CaP cell lines and markedly inhibited occurrence and progression of CaP in TRAMP model.

Method: Fluorescence polarization, ^{3}H-labeled androgen displacement, PSA measurement and cell culture assays were performed to determine the affinity of PMCol with androgen receptor both *in vitro* as well as in cell culture. DCF and DNA assays were performed to determine the anti-oxidant and growth inhibitory activity of PMCol against androgen-dependent and androgen-independent CaP cells. Oral formulation of PMCol was standardized for animal studies. LC-MS protocol was standardized for determining Pharmacokinetics (PK) of PMCol. Scale up synthesis for GMP grade material has been standardized. Tolerance of PMCol in 14 and 28 day mice, 14 and 28 day rat and 14 and 28 day dog studies were determined. Genotoxicity battery and bacterial reverse mutation assays were also performed. Prostate tumor progression in TRAMP animals was determined by periodic tumor palpation and pathological examination after sacrifice.

Results: PMCol binds to the androgen receptor both *in vitro* and in LNCaP androgen-dependent human CaP cells. The binding is weaker than is standard of care (SOC) anti-androgen bicalutamide (Casodex, Astra Zeneca). However, unlike bicalutamide, PMCol inhibited the growth of both androgen-independent and androgen-dependent human CaP cells in culture. PMCol was formulated in PEG-400 or in 1% methylcellulose for oral administration. The serum level of the drug peaked at 15 minute after oral administration and C_{max} was 40 μg/ml after administering a single oral dose of 100 mg/kg. The MTD in mice and rats was 2 g/kg and NOAEL at 1g/kg. In dogs, MTD was 0.5 g/kg and NOAEL at 250 mg/kg in 28 day continuous treatment. PMCol is significantly more effective than is anti-androgen flutamide in delaying tumor progression in TRAMP animals.

Conclusion: Based on these results, PMCol is now being submitted to FDA as an Investigational New Drug (IND) for Phase I/IIa trial in castrate-refractory CaP.

(Dr. Basu and Dr. Wilding have substantial financial interest in Colby Pharmaceutical Company, which has in-licensed PMCol for commercialization.)

Keywords: anti-oxidant, anti-androgen, prostate cancer

232 Metabolic Adaptation and Intracrine Androgen Synthesis in Metastatic Prostate Cancer: Mechanisms for Castration-Resistant Tumor Growth

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Therapy for advanced prostate cancer centers on reducing systemic androgens and blocking activation of the androgen receptor (AR). Despite anorchid serum androgen levels, nearly all patients develop castration-resistant prostate cancer (CRPC). We hypothesized that steroidogenesis within prostate tumors leads to the maintenance of intratumoral androgens and contributes to the development of CRPC. Using mass spectrometry and quantitative reverse transcription–PCR, we quantitated androgen levels and transcripts encoding steroidogenic enzymes in benign prostate tissue, untreated primary prostate cancer, metastases from patients with CRPC, and xenografts derived from CRPC metastases. Testosterone levels within metastases from anorchid men [0.74 ng/g; 95% confidence interval (95% CI), 0.59–0.89] were significantly higher than levels within primary prostate cancers from untreated eugonadal men (0.23 ng/g; 95% CI, 0.03–0.44; $P < 0.0001$). Compared with primary prostate tumors, CPRC metastases displayed alterations in genes encoding steroidogenic enzymes, including up-regulated expression of FASN, CYP17A1, HSD3B1, HSD17B3, CYP19A1, and UGT2B17 and down-regulated expression of SRD5A2 ($P < 0.001$ for all). Xenografts of CRPC propagated in castrate murine hosts maintained intratumoral androgen levels essentially equivalent to those passaged in eugonadal animals. Metastatic prostate cancers from anorchid men express transcripts encoding the full co mplement of androgen-synthesizing enzymes and contain intratumoral androgens at concentrations capable of activating AR target genes and maintaining tumor cell survival. Together, these data indicate that intracrine steroidogenesis may permit tumors to circumvent low levels of circulating androgens, and identifies important targets for therapy designed to inhibit intracrine steroidogenic pathways within the prostate tumor microenvironment.

Keywords: prostate, androgen, metastasis

233 Irx5 as a Target of Prostate Cancer Therapeutics

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1,25-Dihydroxyvitamin D(3) [1,25(OH)(2)D(3)], the most active metabolite of vitamin D, has significant antitumor activity in a broad range of preclinical models of cancer. In this study, we show that the Iroquois homeobox gene 5 (Irx5) is down-regulated by 1,25(OH)(2)D(3) in human prostate cancer samples from patients randomly assigned to receive weekly high-dose 1,25(OH)(2)D(3) or placebo before radical prostatectomy. Down-regulation of Irx5 by 1,25(OH)(2)D(3) was also shown in the human androgen-sensitive prostate cancer cell line LNCaP and in estrogen-sensitive MCF-7 breast cancer cells. Knockdown of Irx5 by RNA interference showed a significant reduction in LNCaP cell viability, which was accompanied by an increase in p21 protein expression, G(2)-M arrest, and an increase in apoptosis. Using LNCaP cells overexpressing a dominant negative form of p53, we also concluded that the apoptotic effect of Irx5 RNAi is partially dependent of the wildtype p53 function.

The biological function of Irx5 in cell viability and proliferation is associated with its regulation of gene transcription. Using an Irx5 target gene promoter-reporter assay, we saw that Irx5 acted as a transcriptional activator in LNCaP cells, and a repressor in Hek293T cells. While Irx5 RNAi induced apoptosis in LNCaP cells, it did not alter the growth and proliferation of Hek293T cells. In contrast, overexpression of a full length Irx5 expression vector had no effect on LNCaP cells, but induced growth inhibition and apoptosis in Hek293T cells. The inhibitory effect of Irx5 was dependent on the homeobox domain and C-terminal catalytic domain of the protein in Hek293T cells. These two domains of Irx5 also play important role in transcriptional activation and repression in LNCaP and Hek293T cells, respectively. More recently, we identified that Irx5 interact with chromatin remodeling enzyme EZH2 in both LNCaP and Hek293T cells. Currently, we are testing the molecular functions of Irx5 in additional human cancer cell lines including breast, cervical, liver and lung. Based on the Irx5 target gene promoter-reporter assay in LNCaP cells, we are in the process of developing a high-throughput platform of identifying small-molecule Irx5 inhibitors. The clinical compound library (Dr. J.O. Liu at Johns Hopkins School of Medicine) that is consisted of ~3,000 compounds that are either FDA-approved or in phase II/III clinical trials will be used for the screening.

These findings suggest that Irx5 may represent an important molecule regulating the prostate cancer apoptosis, and the identification of Irx5 small molecule inhibitor, Irx5 target genes and Irx5 interacting proteins may identify novel therapeutic strategy of treating prostate cancer patients.

Keywords: Irx5, prostate, vitamin D

234 FGF Signaling as a Target in Prostate Cancer Progression

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In prostate cancer, androgen-blockade strategies are commonly used to treat osteoblastic bone metastases, but responses to these therapies have short duration, and eventually tumors progress. The mechanism for androgen-independent progression in bone is unclear. We established two prostate cancer xenografts (MDA PCa 118a and MDA PCa 118b) from an osteoblastic bone metastasis in a man with castration-resistant prostate cancer. These xenografts are androgen-receptor negative and can grow in both castrated and intact male mice. Also, these cells induce a robust osteoblastic reaction in bone and in the subcutis of immunodeficient mice. We found that these cells display a pattern of gene expression similar to that of the osteolytic prostate cancer cell line PC3, in that both MDA PCa 118 and PC3 express bone morphogenetic proteins, Wnts, and endothelin-1 as well as the Wnt inhibitor dickkopf-1 and the osteoclast-activating factor parathyroid hormone-related peptide, suggesting that other factors are involved in MDA PCa 118–induced osteogenesis. In a gene array analysis, we identified FGF9 as being overexpressed in the xenografts relative to other bone-derived prostate cancer cells of mildly blastic (MDA PCa 2b) or lytic (PC3) phenotypes and discovered that FGF9 induced osteoblast proliferation and new bone formation in a bone organ assay. We also found that the MDA PCa 118–induced osteoblast proliferation was blocked by FGF9 antibody in vitro. More important, mice treated with FGF9 neutralizing antibody developed smaller MDA PCa 118 bone tumors and reduced bone formation ($P = 0.011$ and $P = 0.0057$, respectively). Finally, we found positive FGF9 immunostaining in prostate cancer cells in 24 of 56 primary tumors derived from organ-confined prostate cancer and in 25 of 25 bone metastasis cases studied. Findings were confirmed by RT-PCR analysis of RNA obtained by laser capture microdissection of normal prostate epithelial cells and prostate cancer epithelial cells derived from two bone metastases. Collectively, these results suggest that FGF9 contributes to MDA PCa 118–induced osteogenesis and may participate in the osteoblastic progression of prostate cancer in bone. Recent experimental studies of prostate cancer in mouse models indicated that activation of prostate epithelium–stroma communication through FGF signaling leads to tumor development and progression [1, 2] and that ablation of FRS2α, a gatekeeper that mediates FGFR downstream signals, in prostate epithelial cells significantly inhibits prostate cancer development in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice [3]. Taken together, these observations provide strong evidence implicating FGF signaling in prostate cancer development. These observations lead us to hypothesize that pharmacologic blockade of the FGFR signaling pathway will alter the biology of human prostate cancer in a manner that will be therapeutically beneficial for patients with prostate cancer. We will thus perform a proof of principle clinical study with the FGFR inhibitor TKI 258 (a receptor tyrosine kinase inhibitor (TKI) with strong activity against FGFR1-3 ($IC50 < 40$ nM) (Novartis Pharmaceuticals Corporation, personal communication and [4, 5]) in selected patients with castration-resistant prostate cancer and bone marrow infiltration. This study will assess safety, pretreatment, and postreatment values in markers of response in the bone microenvironment (by using serial bone marrow biopsies), and changes in markers of bone remodeling. We will thus correlate changes in the markers of response with the clinical course of evaluable patients. The results will serve as the foundation for the development of candidate predictive markers and further therapies based on targeting the FGF pathway.

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Keywords: prostate cancer, FGF, therapy

235 Finasteride Attenuates the Association of Elevated C-Peptide With Increased Risk of High Grade Prostate Cancer: The Prostate Cancer Prevention Trial (PCPT)

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Metabolic abnormalities (including hyperinsulinemia) and obesity have been associated with increased cancer risk and/or worse cancer prognosis in several studies. C-peptide is a biomarker of pancreatic insulin production and higher levels are suggestive of hyperinsulinemia, which may lead to unfavorable downstream events, including up-regulation of the pAKT and mTOR pathways within neoplastic cells. Our recent laboratory research (eg. Association of diet-induced hyperinsulinemia with accelerated growth of prostate cancer xenografts. J Natl Cancer Inst. 2007 Dec 5; 99:1793-800) suggests a role for insulin in prostate cancer (PCa) biology. Having documented the presence of insulin receptors on PCa biopsy tissue, we recognized that there are opportunities for related translational research in both treatment and prevention contexts.

With respect to treatment, Phase I trials of insulin receptor–targeting agents are underway, and other modalities such as lifestyle modifications or metformin therapy are also being examined as strategies to reduce high insulin levels that may accelerate proliferation of androgen-independent PCa.

We report here early results in the prevention context, carried using the biorepository of the PCPT. The primary results of Phase III trials provide important evidence about the overall effectiveness of a drug on disease prevention or treatment with immediate clinical applications, but work with associated biorepositories can provide additional information concerning cancer biology and also can examine whether there are subgroups of individuals for whom the investigative drug may be particularly beneficial or harmful. The PCPT was a Phase III, randomized, double-blinded placebo controlled trial of the drug finasteride for the primary prevention of PCa. The goal of our research was to investigate whether C-peptide is associated with PCa risk, and in particular whether the risk differed for men taking finasteride vs. placebo. We used specimens from 1803 prostate cancer cases and 1797 controls. Case or control status for all participants was determined by sextant biopsy and central pathology review. C-peptide was assayed by ELISA. Higher vs. lower C-peptide concentrations were associated with a nearly two-fold increased risk of high-grade PCa (Gleason $\geq$ 7), but only among men in the PCPT placebo group. The multivariate high grade PCa cancer odds ratio for the fourth vs. first quartile of C-peptide was 1.90 (95% CI, 1.2-3.0). These estimates were adjusted for age, race/ethnicity, family history of PCa, use of insulin, body mass index and smoking. The data imply that hyperinsulinemia increases risk of high grade CaP, and that finasteride attenuates this risk. If confirmed, these findings have obvious clinical relevance.

Keywords: prostate cancer, finasteride, C-peptide, insulin

236 Treating Advanced Prostate Cancer by Disrupting the Tumor Ecosystem

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Tumors can be defined as ecosystems in which the various host and cancer cells (species) interact with their environment. A key component of the tumor ecosystem is tumor associated macrophages (TAMs), which can be considered an invasive species within the tumor ecosystem that support the growth of the cancer cells. TAMs offer an attractive target for cancer therapeutics. We have identified monocyte chemoattractant protein – 1 (MCP-1, CCL2) as a novel and potent regulator of prostate cancer tumorigenesis because it is the key mediator of the attraction of TAMs to the tumor sites.

CCL2 is a member of the CC chemokine family and was originally described for its sentinel role in regulating monocyte / macrophage migration to sites of inflammation and wound repair. CCL2 has been shown to be an active mediator of tumorigenesis and metastasis of several cancers, including roles in regulating the migration and proliferation of breast, cervical, pancreatic, multiple myeloma, and prostate cancer cells. We have demonstrated:

- Utilizing tissue procured through the Prostate SPORE Rapid Autopsy Program at the University of Michigan, we determined that CCL2 was significantly overexpressed in bone metastases as compared to normal tissues as well as metastases from other sites.
- Analysis of CCL2 secretion by several constituents of the bone-tumor microenvironment by ELISA revealed that the bone marrow endothelial cells secrete significantly higher basal levels of CCL2 compared to PCa cells, osteoblasts, and adipocytes.
- Analysis of human tumors from metastases as well as from *in vivo* preclinical models reveals a high percentage of infiltrating macrophages.
- CCL2 stimulates the maturation of osteoclasts in the bone tumor microenvironment.
- Inhibition of CCL2 *in vivo* leads to significant inhibition of tumor growth in multiple preclinical models of prostate cancer and other cancers.

CCL2, therefore, mediates tumor growth through effects on cancer cell proliferation and migration, osteoclast maturation, and macrophage recruitment and education. Strategies that inhibit CCL2 are in active development and have entered into clinical trial. Proof of principle trials are planned through the SPORE mechanism. Because this type of therapy is targeted against various aspects of the microenvironment, trials across disease sites are planned through SWOG.

Keywords: therapy, prostate, monocyte

237 MDV3100, A Novel Androgen Receptor Antagonist for Castration Resistant Prostate Cancer (CRPC)

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Background: CRPC is characterized by persistent, high level androgen receptor (AR) expression and remains AR-dependent in model systems. MDV3100 is a novel small molecule AR antagonist that unlike bicalutamide, inhibits AR function by imparing nuclear translocation, DNA binding, has no agonist activity when AR is overexpressed, and induces regressions of established human LNCaP-AR xenografts in a dose dependent manner. In July 2007, a multi-center first-in-man Phase 1-2 trial was initiated to determine safety, pharmacokinetics (PK), and antitumor activity including changes in prostate-specific antigen (PSA), bone and soft tissue metastases, circulating tumor cell (CTC) number (CellSearch^{TM} (Veridex, LLC)) and in selected patients, fluorodeoxyglucose (FDG) and fluorodihydrotestosterone (FDHT) uptake by positron emission tomography (PET).

Methods: Pts were administered MDV3100 orally, once daily, starting at 30 mg with sequential escalations in cohorts of 3 pts. Enrollment was expanded at 60 mg and above once the safety of a dose was established. PK parameters for the dose-escalation cohorts were estimated using a 2-compartment model. Outcomes were reported using the Prostate Cancer Working Group Guidelines (JCO 26:1148, 2008).

Results: Accrual through the 240 mg/day dose level has been completed, and is ongoing at 360 mg/day. There have been no reports of serious adverse events attributable to study drug. PK including Cmax, Ctrough, and AUC24h are linear and the half-life is approximately one week. At 12 weeks, PSA declines of 90% or greater were observed in 9% (2/22), 13% (3/23) and 29% (8/28) of patients treated at 60, 150 and 240 mg per day, respectively, associated with stabilization of disease by imaging. ***CTC.*** For the 60 and 150 mg dose levels, favorable (Fav, 4 or less) and unfavorable (Unfav, 5 or more) counts were noted in 58% (25 of 43) and 42% (18 of 43) of cases. Following treatment, 92% of patients retained Fav counts, while 33% and 56% at 60 and 150 mg daily, converted from an Unfav to a Fav category. ***FDG and FDHT PET.*** Individual lesions are scored as 1, negative for tumor; 2, probably negative; 3, equivocal; 4, probably positive; and 5, certainly positive, and SUVmax recorded for lesions scored as 4 or 5. Four patterns were observed for the two tracers: concordant, glycolysis (FDG) predominant, AR (FDHT) predominant, and mixed, with at least one concordant lesion in all patients. To date, 8 pts have had FDG and FDHT PET at baseline of whom 4 have had 1 or more follow-up scans. All 8 had abnormal FDHT accumulation at baseline while 4 of 4 with follow-up scans had no FDHT accumulation. Decreases in FDG uptake in tumor were also observed. **Conclusions:** MDV3100 has been well-tolerated with encouraging anti-tumor activity assessed by: post-therapy PSA declines, imaging, the proportion of patients who are continuing on treatment, and biomarker changes including CTC, FDG and FDHT PET. The observed dose-response trends suggest these proportions may increase as higher doses are explored. Accrual is continuing. A structured Academic-Industry collaboration through the PCCTC can partner to develop promising therapies rapidly.

Supported by Medivation, Prostate Cancer Foundation, PCCTC and MSKCC SPORE in prostate Cancer.

Keywords: prostate cancer, androgen receptor, circulating tumor cells, PET imaging

238 GLIPR1: A Novel Tumor Suppressor Protein With Therapeutic Potential

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Our previous studies have defined GLIPR1 as a secreted, cytostatic/pro-apoptotic tumor suppressor protein that is down-regulated during prostate cancer progression through epigenetic mechanisms. Mechanistic studies have shown that GLIPR1 manifests tumor suppressor functions through coordinated cell type specific activities, including direct, tumor cell selective, pro-apoptotic activities mediated through reactive oxygen species (ROS)-c-jun-NH_2 kinase (JNK) signaling (Li et al., Cancer Res 68: 434, 2008).

Additional studies showed that GLIPR1 expression leads to suppression of Specificity Protein 1 (Sp1) and specific Sp1 regulated target genes including, c-myc and MnSOD. GLIPR1-mediated down-regulation of Sp1 and c-myc results in suppression of cell cycle related proteins including cyclin D1, cyclin A, and cdc25A. Interestingly, down-regulation of c-myc and MnSOD may also underlie GLIPR1 mediated induction of ROS and pro-apoptotic ROS-JNK signaling.

We have documented pro-apoptotic, anti-angiogenic, and immunostimulatory activities of adenoviral vector mediated in situ GLIPR1 gene therapy in an orthotopic mouse model of prostate cancer (Satoh et al., Hum Gene Ther 14: 91, 2003). These preclinical studies led to an ongoing neoadjuvant Phase I/II gene therapy clinical trial in which AdGLIPR1 is being tested by direct intratumoral injection prior to radical prostatectomy (IND #13033).

To further analyze the autocrine and paracrine activities of GLIPR1 we generated specific forms of recombinant GLIPR1 protein including a protein with a deletion of the transmembrane domain (GLIPR1-ΔTM). GLIPR1 and/or GLIPR1-ΔTM treatment results in tumor cell selective growth arrest/apoptotic cell death in multiple prostate cancer cell lines in vitro. Further preclinical studies using VCaP and/or PC-3 orthotopic prostate and bone metastasis xenograft models demonstrated that GLIPR1-ΔTM suppressed tumor growth when administered intratumorally or intraperitoneally. Significantly increased tumor cell apoptosis and reduced tumor associated angiogenesis were also documented.

Keywords: GLIPR1, prostate cancer, protein therapy

239 Tissue Effects of Selenium and Vitamin E in Prostate Cancer

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In two phase III trials, L-selenomethionine (selenium) and alpha-tocopherol (vitamin E) were shown in secondary analyses to reduce prostate cancer incidence. In a randomized, placebo-controlled phase IIA study correlative to the ongoing Selenium and Vitamin E Cancer Prevention Trial (SELECT), we sought to identify differentially expressed genes and to characterize the effects of selenium and vitamin E in normal epithelium, stroma, and tumor tissue.

Evaluated were 39 men with prostate cancer who were treated between diagnosis and prostatectomy (not >6 weeks) with SELECT levels of oral selenium (200 μg) and vitamin E (400 mg) daily. Laser-capture microdissection coupled with microarray was used to study ex vivo prostatectomy biopsy specimens and validated by real-time polymerase chain reaction. To identify genes that could discriminate between treatments and/or tumor types, we fit an ANOVA model. Pair-wise comparisons and contrasts were performed to assess differences between groups. The false-discovery rate (FDR) was estimated using a beta-uniform mixture model.

Normal epithelium, where 2109 genes were differentially expressed, was most affected by selenium (63%); stroma, where 2051 genes were differentially expressed, was most affected by vitamin E (66%); and tumor tissue, where 587 genes were differentially expressed, was most affected by the combination (56%) (FDR, 2%). Overall, we identified gene groups implicated in specific processes and major nodes of interaction in 11 networks and achieved a Spearson's correlation coefficient of 0.87 in tests of representative genes from each cell type differentially expressed.

Almost 600 differentially expressed genes were identified in all three tissue types and linked to specific molecular processes and characterizing networks. Results delineate the cell-type specific and zone-specific tissue effects of selenium and vitamin E. With these results, this model of tissue interrogation, the first of its kind, proves its efficiency, its feasibility, and its hypothesis-generating potential.

Keywords: prostate cancer, selenium, vitamin E

240 5α-Reductase Inhibition in Intermittent Androgen Deprivation Therapy of Prostate Cancer

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Our recent studies showed that androgen-responsive gene U19 (Up-regulated 19) is a tumor suppressor. Interestingly, these genes are supra-induced by the 5α-reductase inhibitor finasteride during the regrowth of a regressed prostate but not in the full-grown prostate naïve to androgen deprivation. These observations led to our research hypothesis: that testosterone (T) is more potent than DHT in the induction of tumor suppressive androgen-response genes during the regrowth of regressed prostate tumors. Blocking T to DHT conversion by 5α-reductase inhibitor during the off-cycle (when T is recovering) supra-induces tumor suppressive androgen-response genes and enhances the efficacy of intermittent androgen deprivation therapy (IADT). If the above hypothesis is correct, IADT plus 5α-reductase inhibitor should be used to enhance the efficacy of intermittent androgen deprivation therapy for prostate cancer patients.

Using LNCaP prostate cancer xenograft model, we reported that IADT with finasteride indeed provides the most favorable tumor growth kinetics and survival rate compared to both continuous androgen deprivation and parallel intermittent androgen deprivation. We have tested the effect of finasteride on several androgen-response genes and ETS genes and found that finasteride enhanced the expression of tumor suppressive androgen-response gene U19 in LNCaP xenograft tumors during the off-cycle in IADT. This supports our hypothesis that T is more potent than DHT in the induction of tumor suppressive androgen-response genes during the regrowth of regressed prostate tumors. Using tumor size as the trigger point for the switch from off-cycle to on-cycle, we also showed that the first off-cycle in IADT could be prolonged twice by finasteride or dutasteride in the LNCaP xenograft tumor model. Our current effort is trying to optimize the interval of the off-cycle and to determine if supra-induction of U19 expression can be used to guide the switch from the off- to on-cycle in IADT.

Keywords: prostate cancer, intermittent androgen deprivation therapy, 5a-reductase inhibitor

241 Role of Akt-Foxo1a Signaling in Synergistic Growth Inhibition of Squamous Cell Carcinoma of the Head and Neck by EGFR Tyrosine Kinase Inhibitor Erlotinib and Green Tea Polyphenol EGCG

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The Akt-Foxo1a signaling is emerging as an important mediator of cell cycle arrest and apoptosis. Foxo1a is a direct substrate of Akt. Akt-mediated phosphorylation of Foxo1a exports it from the nucleus and thereby inactivates its activity, which otherwise regulates the transcription of genes responsible for cell cycle arrest (p21, p27 and Cyclin G2) and apoptosis (Bim and Puma).

In the present study, we have shown that EGFR tyrosine kinase inhibitor erlotinib, and green tea constituent EGCG, have synergistic anti-tumor activity in squamous cell carcinoma of the head and neck (SCCHN) as evidenced by increased apoptosis and inhibition of xenograft growth in nude mice. Propidium iodide and Annexin V-PE staining was performed to study the cell cycle distribution and apoptosis respectively. Expression patterns of cell cycle and apoptosis regulatory proteins were studied by Western blotting. Lenti-virus based expression system using short hairpin RNA (shRNA) was employed to ablate the expression of the specific protein. Synergistic cell growth inhibition by the combination of EGCG and erlotinib was associated with significantly greater inhibition of pEGFR and pAKT, increased activation of caspases 9, 3 and PARP than the inhibition induced by EGCG or erlotinib alone. To further explore the mechanism of these observations, we found that treatment of SCCHN with erlotinib time-dependently increased (3-15 fold) the expression of cell cycle regulatory proteins, p21 and p27 and apoptosis regulatory protein Bim. Erlotinib also induced Puma in some cell lines. Cells underwent G1 arrest with very little apoptosis. In contrast, EGCG alone at a concentration of 30 μM had very little or no effect on the expressions of these proteins varying among the cell lines. However, simultaneous use of EGCG with erlotinib synergistically increased apoptosis and strongly inhibited (50-80% reduction) the expression of p21 and p27 without affecting the induction of Bim and Puma. In addition, erlotinib induced both Foxo1a and p53 transcription factors in SCCHN cell lines. In some cell lines, EGCG also induced these proteins to some extent. Ablation of p53 by shRNA suggested that p53 is dispensable for the expression of Bim, p21 or p27 and for the synergistic apoptotic effect.

Taken together, our results suggest that erlotinib induced both checkpoint proteins p21 and p27 and apoptosis regulatory proteins Bim and Puma probably by activating the Foxo1a transcription factor through inactivation of Akt. EGCG synergistically increased the anti-tumor activity of erlotinib by inhibiting the checkpoint proteins p21 and p27 without affecting the expression of Bim and Puma. (Supported by NCI P50 CA128613).

Keywords: head and neck cancer, Erlotinib, green tea polyphenols

242 Cetuximab and Bevacizumab in Patients With Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma

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Background: Epidermal Growth Factor Receptor (EGFR) and Vascular Endothelial Growth Factor (VEGF) represent therapeutic targets in SCCHN. Cetuximab, an IgG1 monoclonal antibody against EGFR, has single agent activity. Upregulation of VEGF has been associated with cetuximab resistance, thus, combined targeting may enhance anti-tumor activity. We designed a phase II trial of bevacizumab, an anti-VEGF humanized monoclonal antibody, with cetuximab to test this hypothesis.

Methods: Eligible patients have recurrent or metastatic SCCHN, measurable disease (RESIST), ECOG performance status (PS) 0-2, and no history of bleeding or thrombosis. Up to 1 regimen (chemotherapy, but not EGFR inhibitors) for recurrent or metastatic disease and prior chemoradiotherapy with curative intent are allowed. Treatment consists of weekly cetuximab, 250 mg/m^2 (after a loading dose 400 mg/m^2) and bevacizumab, 15 mg/kg given intravenously every 21 days, until disease progression. The primary endpoint is the objective response rate. The one-stage design (sample size 48) included an interim safety analysis after 12 patients enrolled. Specific biomarkers relating to EGFR and VEGFR signaling as well as angiogenesis, proliferation and apoptosis are to be evaluated in tumor tissue and blood samples.

Results: With 22 eligible patients, the median age is 60 years (range 33-92); male 16; PS 0, 6 pts; PS 1, 14 pts and PS 2, 2 pts. All pts have had prior RT and 21 had chemotherapy. Best response in 20 evaluable patients: 3 (15%) partial response, 11 (55%) stable disease, and 6 (30%) progressive disease. Grade (G) 3 adverse events included: hypertension, 1; stomatitis, 1; rash, 3; dysphagia, 2; dehydration, 1; hypophosphatemia, 1; and fatigue, 1. G 4 adverse events: proteinuria, 1. One pt died of aspiration pneumonia, with possible cardiac ischemia of uncertain relationship to study drugs. No hemorrhagic events were observed.

Conclusions: Preliminary results show that cetuximab and bevacizumab is an active regimen in SCCHN. There has been no associated bleeding. Study accrual continues and a biomarker analysis is planned. (Supported by U01 CA099168-01 and P50 CA097190)

Keywords: head and neck cancer, cetuximab, bevacizumab

243 Imaging VEGF Receptors in Tumor Angiogenesis With Novel scVEGF-based SPECT, PET, and NIRF Tracers

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Angiogenesis is a fundamental feature of tumor growth that might be used for diagnostic purposes. Angiogenesis is a target of massive drug development efforts, which have resulted in several FDA-approved drugs. As anti-angiogenic treatments are quite expensive and are not without serious side effects, there is a great need for better diagnostic tools to guide the efficient and safe use of these new drugs in cancer patients. In particular, clinical use and development of anti-angiogenic drugs and combination therapeutic regimens needs imaging tracers that can follow the primary therapeutic target in angiogenic vasculature, receptors for vascular endothelial growth factor (VEGF).

To image not just prevalence, but functional activity of VEGF receptors, we have recently developed a novel class of scVEGF-based tracers for SPECT, PET, and NIRF imaging modalities. scVEGF is a single-chain VEGF, combining two 3-112 fragments of $VEGF_{121}$, expressed with N-terminal 15-aa Cys-tag, a cysteine-containing tag for site-specific conjugation of various "payloads". A robust scalable procedure for purification of bacterially-expressed scVEGF has been developed and protein appears to be remarkably stable. We have site-specifically radiolabeled scVEGF with ^{99m}Tc for single photon emission computed tomography (SPECT), derivatized scVEGF with chelators for positron emission tomography (PET) and SPECT with various radionuclides, and with fluorescent dyes for near-infrared fluorescent (NIRF) imaging. Experiments in mouse tumor models revealed selective accumulation of scVEGF-based tracers mediated by VEGF receptors in endothelial cells in tumor and surrounding host vasculature, starting from very early stages.

Our leading translational candidate is a SPECT tracer, scVEGF directly radiolabeled with ^{99m}Tc (scVEGF/Tc) through a simple "shake and bake" procedure with a tin-tricine reagent. The procedure yields a tracer with a specific activity of 150-200 mCi/mg (4.2 to 5.6 mCi/nmole protein). SPECT imaging and autoradiography established distinctively non-homogeneous accumulation of scVEGF/Tc in tumor. With respect to the muscle tissue, tumor uptake was 7 to 10-fold greater in the angiogenic tumor rim and 3 to 4-fold greater in the central area. The organs with the highest uptake were kidney (~50 %ID/g) and liver (~6 %ID/g). scVEGF/Tc was cleared from blood rapidly with <5 %ID/g at 1 hour post-injection, but remained stable and capable of binding VEGF receptors throughout 1 hour of circulation.

SPECT imaging with scVEGF/Tc has been used for imaging effects of FDA-approved VEGFR-2 kinase inhibitor, Sutent (Pfizer). Sutent treatment decreased specific (normalized per volume) accumulation of tracer in orthotopic xenografts of MDA231luc human breast carcinoma cells in SCID mice. Importantly, tracer uptake in angiogenic tumor rim was decreased less than in tumor center, suggesting a possible mechanism of resistance to Sutent and Sutent-like inhibitors.

In summary, scVEGF/Tc tracer for imaging functionally active VEGF receptors has been validated in pre-clinical models and is ready for translation into clinical development.

Keywords: VEGF receptors, VEGF-based imaging tracers, SPECT, PET, NIRF imaging

244 The Role of Lung-Derived Regulatory T Cells in K-Ras-Driven Lung Tumorigenesis

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National Cancer Institute

Background: K-Ras mutations occur in up to 30% of human lung adenocarcinomas [1] and occur almost exclusively in patients with a history of smoking. In preclinical mouse models of lung cancer, K-Ras mutations are necessary for tobacco carcinogen-driven lung tumorigenesis, and are sufficient to cause lung adenocarcinomas in transgenic mice [2]. Because K-Ras mutations confer resistance to commonly used cytotoxic chemotherapies and targeted agents [3], effective therapies that target K-Ras are needed. Inhibitors of mTOR such as the immunosuppressant rapamycin can prevent K-Ras mediated murine lung tumorigenesis [4,5], suggesting that components of the immune system such as Foxp3+ regulatory T cells (Treg) might be important.

Methods and Findings: Lung tumorigenesis was studied in three different murine models of lung tumorigenesis that depend on mutant K-Ras; a tobacco carcinogen-driven model, a syngeneic inoculation model and a transgenic model. Splenic and lung-associated T cells were studied using flow cytometry and immunohistochemistry. Exposure of A/J mice to the tobacco-carcinogen NNK that causes K-Ras mutations [6] tripled the fraction of lung-associated Treg prior to tumor development. At physiologic levels, rapamycin prevented this increase, coincident with a 90% decrease in lung tumors. In A/J mice inoculated with syngeneic lung adenocarcinoma cells that were resistant to the anti-proliferative effects of rapamycin, rapamycin neither inhibited tumor growth nor decreased Treg. In contrast, administration of an anti-CD25 antibody to inoculated mice decreased Treg and prevented lung tumorigenesis by 80%. Transgenic mice that express mutant K-Ras that had been crossed to mice that bear a loss of function mutation in Foxp3+ also developed 75% fewer lung tumors.

Conclusions: These studies show that Treg are required for K-Ras-mediated lung tumorigenesis. Given the availability of rapamycin and antibodies that target Treg, strategies that deplete Treg will be tested clinically to determine if decreasing Treg will inhibit K-Ras driven lung cancer.

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Keywords: lung cancer, K-Ras, regulatory T cells

245 Clinically Relevant Platinum Agents

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Structurally novel platinum compounds, discrete from that of cisplatin and its congeners, also affect target DNA conformation and structure in distinct manners, leading to an altered profile of antitumor activity. The polynuclear drug, BBR3464, has undergone clinical trials. The interactions of this class of drugs with target DNA are distinct from the mononuclear-based cisplatin family and, indeed, unlike those of any DNA-damaging agent in clinical use. The chemical and biological features make these drugs representative of an entirely new structural class of DNA-modifying anticancer agents. BBR3464 remains the only platinum-based drug outside the cisplatin structural class (carboplatin, oxaliplatin, satraplatin, picoplatin) to be tested in humans. The entry into the clinic of a first example of a totally new class of polynuclear platinum-based anticancer drugs is proof of principle of the utility of the approach. The Phase I trials demonstrated a clear pattern of responses in cancers not normally treatable with cisplatin including responses in melanoma, pancreatic and lung cancer as well as breast cancer (in combination with 5-FU). Objective responses in Phase II were verified in relapsed ovarian cancer and non-small cell lung cancer. Pre-clinical studies indicated activity in p53-mutant tumors and a minimal induction of p53 following BBR3464 treatment. Metabolism studies indicated relatively rapid breakdown of the trinuclear structure, raising doubts as to the feasibility of BBR3464 as a clinical agent. New 2nd-generation analogues have been designed that resolve these issues – good metabolic profiles, antitumor activity *in vivo* in human xenografts, high (possibly selective) accumulation in tumor cells and activity in p53-mutant cells *in vitro* present a promising profile for further development.

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Keywords: platinum, clinical agents, new DNA modifying modes

246 Chemoprevention of Lung Carcinogenesis Using Green Tea: A Phase IIB Randomized, Double-blinded, Placebo Controlled Trial of Green Tea Extract and Polyphenon E in Former Smokers With Chronic Obstructive Pulmonary Disease (COPD)

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Many laboratory studies have shown an inhibitory action of green tea or the polyphenolic fraction of green tea in animal models of lung carcinogenesis. Thus, the role of tea drinking as a potential inhibitor of carcinogenesis merits careful evaluation. In our attempt at translating the abundant pre-clinical information and epidemiological data to the human population, we are completing a Phase IIb 3-arm randomized, placebo controlled, double blinded green tea intervention trial among former smokers with chronic obstructive pulmonary disease (COPD) and >= 30 pack-years of smoking history. This population is targeted because they have been identified as having a high prevalence of premalignant dysplasia. Subjects will be randomly assigned to consume daily for six months either a standardized green tea (GT) beverage, or a defined green tea polyphenol (GTP) extract in capsule form, or placebo preparations. The hypotheses to be tested in the proposed research are 1) high consumption of GT or GTP can protect against cellular oxidative damage and 2) high consumption of GT or GTP can modulate the expression of genes involved in proliferation and apoptosis in a population at elevated risk of lung cancer. The primary endpoints will be improvement in markers of oxidative damage in DNA and lipid (levels of 8-OHdG, 8-epi-PGF2, and catalase, superoxide dismutase and glutathione peroxidase activities). The secondary endpoints will be exploratory to assess changes in the gene expression of biomarkers of proliferation (EGFR, PCNA, JUN, FOS, Ki-67) and apoptosis (caspase-3) in induced sputum. In addition, we will seek to determine if there are differences in adherence between the green tea preparation groups. We believe that a program of nutritional intervention by realistic dietary modifications that are effective, safe, and acceptable should be the cornerstone of lung cancer prevention strategy.

Keywords: chemoprevention, lung, green tea

247 Role of MET Receptor Tyrosine Kinase in Small Cell Lung Cancer and Potential for Novel Therapy

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Small cell lung cancer (SCLC) has an overall prognosis of 6%, and novel therapies are desperately needed. Previously targeted therapies against SCLC have not been successful. MET receptor tyrosine kinase and its ligand hepatocyte growth factor (HGF) has been shown to be involved in proliferation, cell motility and migration, invasion, angiogenesis and metastasis of many solid tumors. We show that the expression of MET/phospho-MET and mutations/amplifications of MET are considerably unique in SCLC as compared to other tumors. In particular, we have identified that: 1. As compared to breast, colon, ovarian, and renal cell carcinoma, lung cancer had the highest activated (p-MET) expression. In further analysis of a larger series of lung cancer tumors, there was increased p-MET expression in SCLC as compared to NSCLC (non-SCLC, 65% versus 40%, respectively). 2. Triple-p-MET (reflecting the auto-catalytic/phosphorylation site in MET) was overexpressed in 25% of SCLC, as compared to 7% NSCLC. 3. MET was expressed in the cytoplasm for 100% of SCLC, whereas, p-MET was expressed (63%) in the nucleus of SCLC. 4. The frequency of missense mutations in MET for SCLC was 12% and synonymous SNPs was 38%; however, the functions for these are currently not known. We have identified mutations of MET in the juxtamembrane (JM) and semaphorin (Sema) domains of MET, and showed them to be gain-of-function mutations. Using C. elegans modeling system we have developed for high-throughput analysis of human genetic mutations and function in a multi-cellular microscopic nematode, we show that the JM domain MET mutants caused a dramatic phenotype related to the vulva, and was further activated with nicotine (an important component of cigarette smoke, and implicated in the pathogenesis for SCLC). We have identified that MET can be activated by HGF, and the phosphorylated form can localize to the nucleus. Within the nucleus, phospho-MET can interact with various proteins such as topoisomerase-I and the transcription factor Pax 5. Interestingly, Pax 5 was expressed in SCLC and not NSCLC; whereas, Pax 8 was expressed in NSCLC and not SCLC. We further show that Pax 5 was a transcription factor for the MET gene in SCLC. Utilizing various inhibitors of c-MET pathway, we show synergism of c-MET inhibition with topoisomerase-I inhibition in SCLC. We and other investigators have helped develop/evaluate a number of MET/HGF inhibitors in clinical trials, and phase II trials are being designed for lung cancer. Since SCLC is a very difficult disease to treat, we also provide evidence that combination with c-MET and topoisomerase-I inhibition will be important for the future. Ultimately, studies on MET in SCLC will yield considerable information for individualized therapy against SCLC, and help fight against this devastating illness.

Keywords: MET, mutations, novel targeted therapies, SCLC

248 Targeting mTOR Through Integrated Pathway Approach to Enhance the Therapeutic Efficacy in Lung Cancer

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The Akt/mTOR pathways are activated in many lung cancer patients and play important roles in the control of tumor cell growth and angiogenesis. Recent preclinical research and clinic trials in a number of cancer types have revealed promising results on the beneficial effect of mTOR inhibitors. In order to enhance therapeutic efficacy in lung cancer, our program project has taken an integrated team approach to target the Akt/mTOR axis with both mechanistic and clinic exploitations of the pathways. Four parallel lines of research have been carried out (i) examining the critical role and the prognostic importance of the mTOR pathway in lung cancer in order to directly address the clinical potential of mTOR inhibitors in the treatment of lung cancer, (ii) dissecting the upstream regulatory pathway of mTOR mediated by LKB1 to evaluate its impact on NSCLC response to mTOR inhibitors and taxanes, (iii) investigating the molecular basis for synergy between taxanes and the signal transduction inhibitors (FTI), and (iv) establishing the role of 14-3-3, a mTOR pathway regulatory protein, in lung cancer and 14-3-3-targeted strategy for enhanced sensitivity to mTOR inhibitors. Through these studies, we have discovered an unexpected IRS-independent mTOR-Akt feedback regulatory system, established a novel role of LKB1 in Akt-mediated cell survival, revealed a ternary regulatory protein complex among FTase, microtubules, and HDAC6 and identified that FTase is a master cellular regulator of HDAC6 activity shedding more light into these proteins' interplay for the synergistic effect of FTI and taxanes, and uncovered a novel function of 14-3-3ζ in anchorage-independent growth of lung cancer cells through anoikis avoidance. Our clinical trials have accrued ahead of schedule, and we have completed our Phase I mechanistic combination trial of lonafarnib, a farnesyl transferase inhibitor, with docetaxel. These exciting results provide a molecular basis for the effective clinical use of the taxane/FTI combination, suggest a new combination therapy strategy of blocking both mTOR and Akt in lung cancer, and identify 14-3-3 as an emerging target for the development of novel therapeutic agents for the treatment of lung cancer.

Keywords: mTOR, combination therapy, lung cancer

249 Strategy for Chemoprevention of Lung Cancer With Polyphenon E in a Subset of Current and Nonsmokers

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The major goal of our grant proposal was to conduct a phase II trial to determine the chemopreventive efficacy of Polyphenon E administered orally to former smokers. In 2003, the rational for this trial was based on: 1) epidemiology studies; 2) numerous animal studies which demonstrated that green tea extracts inhibited the formation of lung adenomas in mice when administered in drinking water beginning one week after carcinogen treatment; 3) similar inhibitions observed with green tea extracts in animal models of other tumor types. However, we understood in 2003 that there were several weaknesses in these rationales. A criterion for recruitment of former smokers into the trial was based on the existence of preneoplastic lesions in the bronchial epithelium. The criteria for efficacy were to inhibit the progression of these preneoplastic lesions to carcinomas. The chemoprevention studies in animal models had not examined this type of inhibition for green tea extracts. Also, the lung tumor types examined in animal models was adenocarcinoma whereas the proposed human trial examined the squamous cell carcinoma pathway.

We address these weaknesses with the animal studies conducted in the past 4 years. Polyphenon E administered in the diet was shown to significantly inhibit the progressions of dysplasia to squamous cell carcinomas in the upper respiratory tract of the hamster. Also, dietary Polyphenon E was shown to inhibit late stage progression of lung adenocarcinomas in our A/J mouse model. Of interest, this reduction of average tumor load in the A/J model was due to the inhibition of very large adenocarcinomas observed in the absolute control group. Numerous studies with the A/J mice and various transgenic A/J mice also dramatically showed that dietary Polyphenon E inhibited tumor progression. It should be noted that blood levels of EGCG after treatment of animals with one to two percent of dietary Polyphenon E corresponded to the blood level of EGCG in human subjects after treatment with a dose of 1600 mg of Polyphenon E per day. These results further enhance the rationale for completing the Phase II Polyphenon E trial.

Modifications of the Phase II trial based on clinical data generated in the past 4 years were proposed and approved by the NCI staff. These modifications include criteria for subject enrollment into the trial and measurements of new biomarkers. Levels of C-Reactive Protein in plasma will be used as novel criteria of enrollment into the trial. In addition, we will recruit both current and former smokers into the trial since Polyphenon E has significant anti-inflammatory and anti-oxidant effects in the lung and systemically in both current and former smokers. This phase II clinical trial will provide new information on the efficacy and safety of a common beverage for chemoprevention of lung cancer.

Keywords: polyphenon E, chemoprevention, lung cancer

250 Role of the *TSC1/TSC2* Genes in Lung Cancer

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#The laboratories of D.J.K. and K.-K.W. contributed equally to this work.

Germline *TSC1/ TSC2* mutations cause Tuberous Sclerosis Complex (TSC), a hamartoma syndrome with lung involvement. To explore the potential interaction between *TSC1/TSC2* and *Kras* activation in lung cancer, mice were generated in which *Tsc1* loss and $Kras^{G12D}$ expression occur at low frequency in the lung epithelium. Mice with combined *Tsc1*-$Kras^{G12D}$ mutation had dramatically reduced tumor latency (median survival 12 weeks) in comparison to $Kras^{G12D}$ alone mutant mice (median survival 24 weeks). *Tsc1*-$Kras^{G12D}$ tumors showed consistent activation of mTORC1, and responded to treatment with rapamycin leading to significantly improved survival, while rapamycin had little effect on cancers in $Kras^{G12D}$ alone mice. *TSC1*/*TSC2* loss of heterozygosity was found in 23% of 87 human lung cancer specimens. Six lung cancer cell lines, of 80 studied (7.5%), showed low TSC1/TSC2 expression and a signaling pattern we call TSC null signaling (TNS), indicative of constitutive mTORC1 activation. TSC1/TSC2 reconstitution reversed this pattern and reduced the growth of the cell lines. These data indicate that *TSC1/TSC2* loss synergizes with *Kras* mutation to enhance lung tumorigenesis in the mouse, and suggests that mTOR inhibitors may have unique therapeutic benefit in lung cancer patients with inactivation of TSC1/TSC2 and TNS signaling.

Keywords: lung neoplasm, tuberous sclerosis complex 1 protein, tuberous sclerosis complex 2 protein

251 Strategies in Targeting Notch3 in Lung Cancer

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The Notch receptors are essential for the control of cell fate determination during the development of many multicellular organisms. A member of Notch receptor family, Notch3, is highly expressed in 40% of all resected lung cancers, and inhibitors of this pathway result in the loss of the malignant phenotype *in vitro* and reduced tumor growth in lung xenograft models. Thus, this pathway represents a potentially important target for therapeutic development. Here we report the early results of a targeted strategy to disrupt Notch3 signaling through blocking receptor-ligand interactions. Previous mutational studies involving deletions of several EGF-like repeats indicate putative ligand binding sites, but the exact sequences necessary for Notch3-ligand interaction have not yet been elucidated.

We therefore made an overlapping set of one hundred fifty-five, small peptides (5-13 amino acids in length) representing nearly the entire extracellular domain on the Notch3 receptor and screened them for their potential to induce apoptosis. In our pool of peptides, we found 6 peptides, clustered in two regions of the extracellular domain, that were capable of inducing apoptosis in the Notch3 overexpressing NSCLC cell line, HCC2429. Several lines of evidence, including reduced expression of the Notch3-regulated gene Hey1 after treatment with these peptides, suggest that the apoptotic changes observed were Notch3-dependent. Using immunoprecipitation, we showed direct specific binding of these peptides to the Notch3 ligand JAGGED1, further supporting our hypothesis that these peptides induce apoptosis through interfering with Notch3 and JAGGED1 interactions. We are attempting to determine the structure of the receptor-ligand interaction in this region and developing a high-throughput screen to identify potential therapeutic peptidomimetics. Finally, we have antisera from mice immunized with portions of the receptor extracellular domain inhibit Notch3 activation, and we are in the process of developing blocking monoclonal antibodies.

In summary, our data indicate that these small peptides can inhibit the Notch3 signaling pathway by interrupting receptor-ligand interactions and inducing apoptosis in human lung cancer cells. These peptides could be the basis for Notch3-targeted therapeutic development for lung cancer.

Keywords: Notch3, antibodies, peptides

252 Lung Cancer Chemoprevention With Celecoxib in Ex-Smokers

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Ample preclinical data suggests that the cyclooxygenase-2 (COX-2)/prostaglandin-E2 (PGE2) signaling pathway plays a pivotal role in conferring the malignant phenotype. Produced primarily by the action of cyclooxygenases on free arachidonic acids liberated from membrane phospholipids, overproduction of PGE2, which is predominantly generated by up-regulation of COX-2, is associated with a variety of carcinogenic mechanisms. These mechanisms include abnormal expression of epithelial growth factors, epithelial and microvascular proliferation, resistance to apoptosis, and suppression of antitumor immunity. COX-2 expression has also been shown to be a poor prognostic indicator in non–small cell lung cancer. Previous studies have shown elevated PGE2 levels in the bronchoalveolar lavage fluid of patients with bronchogenic carcinoma. Treatment with chemotherapy leads to increased amounts of COX-2 and PGE2 in non–small cell lung cancer and co-treatment with Celecoxib abrogates the increase in levels of PGE2. In animal models, inhibition of COX-2 and PGE2 synthesis suppresses lung tumorigenesis. Furthermore, results from a pilot, phase IIa trial in high-risk smokers suggest that Celecoxib may modulate SEBM with reduction of PGE2 production, restoration of anti-tumor immunity and reduction of proliferation indices (Ki-67 labeling index, KI-67 LI). These data support the antineoplastic effect of COX-2 inhibitors and provide the rationale for evaluating their potential in the chemoprevention of bronchogenic carcinoma.

Funded by a U01 mechanism, a phase IIb, randomized, placebo controlled, crossover pilot study has been initiated to determine the feasibility of a COX-2 inhibitor (Celecoxib) for chemoprevention of lung cancer in former heavy smokers (age $\geq$ 45, 30 pack years of smoking history and at least one year of smoking cessation) at risk of developing primary and/or second primary lung cancers. Qualified participants have all undergone comprehensive screening with low dose helical CT scan and fluorescence bronchoscopy. Celecoxib (400 mg twice daily) is being evaluated for its impact on cellular and molecular events associated with lung carcinogenesis. A variety of biological specimens, including bronchial biopsies, bronchoalveolar lavage (BAL) fluid and cells, buccal smear, plasma, urine and exhale breath condensate are being collected from subjects pre and post treatment. The primary end point of the study is modulation of Ki-67 Li on bronchial mucosa following six months of treatment. We prescreened 4,470 subjects, actively screened 323 subjects, performed screening bronchoscopy on 142 subjects and randomized a total of 137 subjects. To date 105 subjects have completed 6 months of treatment. Evaluations of a variety of surrogate endpoint biomarkers are currently underway.

Keywords: lung cancer, chemoprevention, Celecoxib

253 The Combination of EGFR Inhibitors With Gemcitabine and Radiation in Pancreatic Cancer

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Gemcitabine-radiotherapy is a standard treatment for locally advanced pancreatic cancer. Recent clinical data have demonstrated that gemcitabine plus erlotinib is superior to gemcitabine alone for advanced pancreatic cancer [1]. Therefore, we investigated the effects of the combination of EGFR inhibitors with gemcitabine and radiation on a pancreatic cancer model. EGFR signaling was analyzed by measuring phosphorylated EGFR ($pEGFR^{(Y845),(Y1173)}$) and AKT ($pAKT^{(S473)}$) protein levels in pancreatic cancer cell lines and tumors. The effects of scheduling on gemcitabine-mediated cytotoxicity and radiosensitization combined with erlotinib were determined by clonogenic survival. *In vivo*, the effects of cetuximab or erlotinib in combination with gemcitabine-radiation on the growth of BxPC-3 tumor xenografts were measured. We found *in vitro* that gemcitabine induced phosphorylation of EGFR at Y845 and Y1173 that was blocked by erlotinib. Treatment of BxPC-3 cells with gemcitabine prior to erlotinib enhanced gemcitabine-mediated cytotoxicity without abrogating radiosensitization. *In vivo*, cetuximab or erlotinib in combination with gemcitabine-radiation inhibited growth compared to gemcitabine-radiation (time to tumor doubling: Gem+RT 19±3 days, Cet+Gem+RT 30±3 days; $p<0.05$, Erl+Gem+RT 28±3 days; $p<0.1$. Cetuximab or erlotinib in combination with gemcitabine-radiation resulted in significant inhibition of $pEGFR^{(Y1173)}$ and $pAKT^{(S473)}$ early in treatment, and $pEGFR^{(Y845)}$, $pEGFR^{(Y1173)}$, and $pAKT^{(S473)}$ by the end of treatment. This study demonstrates a novel difference $pEGFR^{(Y845)}$ and $pEGFR^{(Y1173)}$ in response to EGFR inhibition. These results demonstrate that the EGFR inhibitors cetuximab and erlotinib increase the efficacy of gemcitabine-radiation and support the integration of EGFR inhibitors with gemcitabine-radiation in clinical trials for pancreatic cancer.

This work was supported by 2RO-1 CA078554 and Cancer Center Core Grant 5 P30 CA46592.

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Keywords: EGFR, gemcitabine, radiation

254 Prostacyclin Prevents Murine Lung Cancer Independent of the Membrane IP Receptor: Potential Role of Porstacyclin-Induced Activation of PPARγ

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While tobacco abstinence and smoking cessation are the critical first steps in reducing lung cancer rates, the majority of US lung cancers are diagnosed in former smokers, and to date no effective chemopreventive agents have been discovered. The large at-risk population (current and former smokers) and poor 5-year lung cancer survival rates underscore the need for a better understanding of chemopreventive mechanisms and effective agents. Prostaglandins play an important role in lung tumorigenesis and prostaglandin manipulation has been investigated for lung cancer chemoprevention. Cyclooxygenase (COX) activity generates numerous downstream mediators, including prostaglandins, prostacyclin, and thromboxanes, that can have pro-tumorigenic or anti-tumorigenic effects. Large epidemiologic surveys have shown fewer lung cancers in 'frequent aspirin users.' Human trials evaluating COX inhibition and lung cancer chemoprevention are being conducted, but conflicting data in murine studies evaluating the role of non-specific COX or selective COX-2 inhibition makes interpretation of these results difficult. In addition, studies showing excess cardiovascular events associated with chronic COX-2 inhibitor use raise concerns about long-term safety. One potential cause for these effects may be lower levels of COX-2 mediated production of prostacyclin which has been shown to have atheroprotective properties. Our laboratory has focused on manipulating prostaglandin production distal to the COX enzymes. Prostacyclin (PGI_2), a metabolite of COX activity with anti-inflammatory and platelet inhibitory properties, has been shown to prevent metastases. Previously, we showed that selective pulmonary overexpression of prostacyclin synthase (PGIS) prevents lung tumor formation in response to either chemical carcinogenesis or exposure to tobacco smoke in mice (1,2). These findings have led to an ongoing clinical chemoprevention trial using the prostacyclin analog iloprost in patients at risk for lung cancer. A single receptor for PGI_2 has been described (IP/PGIR), which is a member of the G-protein coupled receptor family, and signals through increases in intracellular cAMP. Studies using IP receptor knockout mice indicate that activation of IP mediates inflammation, vascular homeostasis, and thrombosis prevention. Reports have also shown that PGI_2 and its analogs are ligands for peroxisome proliferator-activated receptors (PPARs), which are members of the nuclear receptor superfamily of regulated transcription factors. The purpose of this study was to determine the contribution of IP versus PPARs in mediating the anti-tumorigenic effects of PGI_2. To determine the role of this receptor in lung cancer chemoprevention, PGIS-overexpressing mice were crossed to mice that lack the IP receptor (IP (-/-)). Carcinogen-induced lung tumor incidence was similar in IP(+/+), IP(+/-) and IP(-/-) mice, and overexpression of PGIS gave equal protection in all three groups, indicating that the protective effects of prostacyclin are not mediated through activation of IP. Since prostacyclin can activate members of the PPAR family of nuclear receptors, we examined the role of PPARγ in prostacyclin's protection against lung tumorigenesis. Iloprost activated PPARγ in non-transformed bronchial epithelial cells and in a subset of human non-small cell lung cancer cell lines (NSCLC). Iloprost-impregnated chow fed to wild-type mice prior to initiation of tumorigenesis decreased lung tumor formation. Administration of iloprost 5 weeks after tumor initiation was equally effective in inhibiting tumor development. Similar to iloprost-treated mice, transgenic animals with lung specific PPARγ-overexpression developed fewer lung tumors. This reduction was not enhanced by administration of supplemental iloprost. Our experiments demonstrate that prostacyclin mediated lung cancer chemoprevention is independent of the IP receptor, and may be mediated by activation of PPARγ. Manipulation of prostaglandin production distal to COX, and/or direct activation of PPARγ by agents such as pioglitazone, represent novel lung cancer chemopreventive strategies, and may also have therapeutic potential.

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Keywords: TNFerade, head and neck cancer, Phase I trial

255 A Novel Approach to Overcome Cisplatin Resistance by HSP90 Inhibition in Head and Neck Cancer Cell Lines

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We have recently discovered that EGFR is activated upon exposure to chemotherapeutic agents such as cisplatin and gemcitabine in head and neck cancer cell lines. This activation of EGFR leads to ubiquitin mediated degradation of the receptor itself causing cellular toxicity and radiation sensitization (1-3). We hypothesized that the receptor degradation is a critical event in drug-induced toxicity and radiation sensitization. In order to test our hypothesis we screened several head and neck cancer cell lines that are either sensitive or resistant to cisplatin. We have found that EGFR is not degraded in cisplatin resistant cell lines. We hypothesized that if we could promote EGFR degradation we could sensitize these cells to cisplatin-mediated cytotoxicity radiosensitization. For this purpose we choose a novel approach toward targeting EGFR using HSP90 inhibition. We choose this approach because it is known that HSP90 is a major chaperone for Erbb-2 which shares major sequence homology with EGFR at the HSP90 binding site. Our preliminary data show that 1) HSP90 is activated by cisplatin only in cisplatin sensitive cells, 2) EGFR and HSP90 interact and can be co-immunoprecipitated and 3) geldanamycin, an inhibitor of HSP90, accelerates the degradation of EGFR in cisplatin resistant cells, leading to both cellular toxicity and significant radiosensitization. These findings demonstrate that EGFR degradation after chemotherapy depends on HSP90. Furthermore, they suggest that the new generation of geldanamycin analogues that are entering the clinic may potentiate cisplatin-mediated cytotoxicity and radiosensitization via EGFR degradation.

Grant support: NIH through the University of Michigan Head and Neck Specialized Program of Research Excellence grant (1 P50 CA97248).

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Keywords: Cisplatin, HSP90, EGFR

256 Premalignant Lesions of the Lung and Calcitriol: A Phase II Study

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Lung cancer is characterized as a multi-step process involving sequential histopathological changes and the accumulation of numerous epigenetic and genetic alterations caused mostly by chronic exposure to tobacco carcinogens. The histopathological steps of carcinogenesis include the conversion of normal epithelium to metaplasia, to dysplasia, to carcinoma in situ, and eventually to invasive carcinoma. The pathways that underlie the histology of this transformation to cancer include proliferation (MCM2), the disruption of apoptosis (associated with a decrease in Bcl-2), the disruption of epidermal growth factor receptor (changes in EGFR) signaling and the disruption of angiogenesis and cell cycle regulation (a decease in P-Akt). These pathway alterations represent potential chemoprevention targets. We have demonstrated that vitamin D (1,25 dihydroxycholecalciferol or calcitriol), a central factor in bone and mineral metabolism, has significant antitumor activity in vitro and in vivo in a variety of murine and human tumor models including lung, squamous cell carcinoma (SCC) and prostate cancer model systems. The ability of calcitriol to induce cell cycle arrest, apoptosis and differentiation at doses without toxicity, make calcitriol an attractive chemo-preventative agent. We will present evidence the the Vitamin D receptor, which is required to transport of calcitriol into the cell nucleus, is present in lung tissue and premalignant lesions. At RPCI, we have developed a lung cancer screening clinic for high risk patients utilizing autofluorescence bronchoscopy and low dose spiral CT of the chest for early detection of lung cancer where patients are screened, followed and biopsies performed for pathologic and biomarker assessment. Therefore, we propose to test the hypothesis that calcitriol will inhibit the progression or incidence of metaplastic or dysplastic lesions in the lung epithelium of a sample of currently smoking, high risk lung cancer patients seen at RPCI in a double blind, placebo controlled clinical trial of calcitriol. The FDA has requested that a toxicity trial of calcitriol be completed in subjects without cancer using a 45 mcg dose at a lower frequency than was proposed in the previously approved Phase II lung cancer prevention trial. Interim result for the proposed “Pilot single-armed pilot study” of 45 mcg of calcitriol, PO, QOW, dosing will continue for 3-months with a defined toxicity monitoring schedule will be presented.

Keywords: calcitriol, chemoprevention, premalignant lung lesions

257 Development of a Transcription Factor Decoy Targeting STAT3: Implementation of a Phase 0 Trial in Head and Neck Cancer

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STAT3 overexpression has been detected in several cancers including head and neck squamous cell carcinoma (HNSCC). Previous studies using intratumoral administration of a STAT3 decoy oligonucleotide that abrogates STAT3-mediated gene transcription in preclinical cancer models have demonstrated antitumor efficacy. In collaboration with the NCI RAID program, we performed toxicology studies in a non-human primate model and developed a clinical formulation of the STAT3 decoy in anticipation of initiating a Phase 0 Trial in HNSCC patients. Three study groups (two monkeys/sex/group) were administered a single intramuscular injection of the STAT3 decoy (or vehicle control) on day 1 and necropsies were performed on days 2 and 15 (1 monkey/sex/group/day). Two intramuscular doses of the decoy were administered (0.8 mg or 3.2 mg) in a volume of 0.9 ml. Tissue and blood were harvested for toxicology and biologic analyses.

The STAT3 decoy-treated animals exhibited behavior that was similar to the vehicle control group. Individual animal body weights remained within 1% of pretreatment weights throughout the study. Hematological parameters were not significantly different between the control and the treatment groups. Clinical chemistry fluctuations were considered within normal limits and were not attributed to the STAT3 decoy. Assessment of complement activation breakdown product (Bb) levels demonstrated no activation of the alternative pathway of complement in any animal at any dose level. At necropsy, there were no gross or microscopic findings attributed to STAT3 decoy in any organ examined. STAT3 target gene expression at the injection site revealed decreased Bcl-X_L and cyclin D1 expression levels in the animals treated with high dose of STAT3 decoy compared to the animals injected with low dose of STAT3 decoy or the vehicle as control.

Based on these findings, the no-observable-adverse-effect-level (NOAEL) was greater than 3.2 mg/kg when administered as a single dose to male and female Cynomolgus monkeys. Assessment of the clinical grade material demonstrated potent antitumor effects and modulation of STAT3 target genes in preclinical HNSCC models. An IND was granted and the IRB-approved study was recently opened to accrual. Analysis of the pre- and post-treatment HNSCC tumor samples will determine whether or not the STAT3 decoy abrogates target gene expression in human cancer. This will be the first clinical study of a STAT3 targeting agent.

Keywords: STAT3, transcription factor decoy, head and neck cancer

258 Role of Estrogen and Estrogen Receptors in Growth and Progression of Non-Small Cell Lung Cancer

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Evidence from our laboratories and others suggests that the estrogen pathway is active in non-small cell lung cancer (NSCLC) and promotes lung tumor growth. Estrogen receptor β is the main form of the estrogen receptor (ER) found in NSCLC tissue samples and cell lines, although in some instances ERα is also detected. Application of β-estradiol to NSCLC cells in culture leads to increased proliferation and heightened expression of genes associated with cell growth such as cyclin D1 and c-myc, with increased release of the angiogenic mediator VEGF. ERβ-specific ligands mimic effects of β-estradiol in NSCLC cell lines, while ERα specific lignds are less effective. ERβ expression has also been found on lung fibroblasts, and application of β-estradiol to these cells increased release of HGF, the ligand for the oncogenic receptor c-Met. Application of β-estradiol also induced activation of c-Src and release of ligands for the EGFR, with enhanced activation of MAPK. Dual inhibiton of the EGFR and ER pathway using the anti-estrogen fulvestrant and the EGFR tyrosine kinase inhbitiors gefitinib or erlotinib enhanced anti-proliferative effects in cell culture and in a lung cancer xenograft model. Aromatase, the rate-limiting enzyme of β-estradiol synthesis, has also been detected in normal and neoplastic lung tissues and cells, with measureable release of estrogen. In a recent report, Niikawa et al. (Clin Cancer Res 14: 4417, 2008) demonstrated 2.2-fold higher intra-tumoral levels of β-estradiol in lung tumors compared to surrounding normal tissues as detected by mass spectrometry, which correlated with expression of aromatase, increased tumor size and increase Ki-67 labeling index in ER-positive lung tumors.

Taken together, these findings suggest that an autocrine loop for estrogen production and proliferative response to estrogen exists in NSCLC tumors that could be mediated by both ERβ and ERα, and the ER pathway may cooperate with the EGFR pathway to achieve these effects. Therapy with anti-estrogens developed for breast cancer therapy has potential for clinical effectiveness in ERα or ERβ-positive lung cancer. In a preliminary pilot Phase I clinical trial of the combination of monthly i.m. fulvestrant (a pure ER antagonist) and daily oral gefitinib (an EGFR tyrosine kinase inhibitor) in 20 evaluable post-menopausal women with advanced NSCLC conducted between 2004 and 2006, the combination was found to be safe with only one grade 4 toxicity, dyspnea, possibly related to treatment. One year overall survival (OS) was 41% in this trial, and OS was 65.5 weeks in a subset of patients with at least 60% nuclear ERβ staining in their tumor tissues. The disease control rate was 75% which included 3 partial responses. Because of low efficacy in NSCLC, gefitinib has since been replaced in the treatment of NSCLC by the EGFR TKI erlotinib. In a second randomized Phase I/II trial, daily oral erlotinib is being combined with monthly i.m. fulvestrant at a 2:1 ratio randomized to erlotinib alone. The trial is enrolling both men and post-menopausal women, and an interim safety analysis in 15 patients showed no unexpected toxicities with the combination compared to erlotinib alone. Because of the high clinical effectiveness that aromatase inhibitors (AIs) have shown in breast cancer, new trials should also be carried out with AIs for lung cancer treatment. A caveat for use of AIs is that estrogen replacement therapy has been shown in some studies to protect healthy female smokers from developing lung cancer, suggesting that there may be some systemic benefits of estrogen before a lung cancer diagnosis. This could be due to P450 enzyme induction, leading to increased de-toxification of tobacco carcinogens. Therefore the ability of AIs to prevent lung cancer in healthy smoking women is uncertain. Initial trials with AIs for lung cancer treatment should be conducted only with ex-smokers or those who have never smoked.

Keywords: lung cancer, anti-estrogen, aromatase inhibitor

259 Biorepository for Validation of Biomarkers for Cervical Cancer Screening: Incorporation of Virtual Slide Repository for Improved Endpoint Determination

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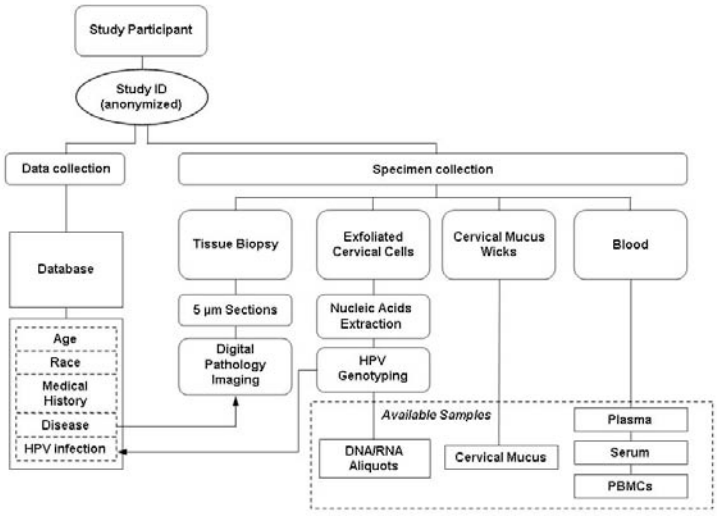
Cervical cancer screening programs in the U.S. are not directed at invasive disease, but at detection of cancer precursors. Human Papillomaviruses (HPV) play an etiologic role in cervical cancer and have been used as screening biomarkers. Recently available HPV vaccines will not eliminate the need for screening as not all types of HPVs associated with cancer are targeted by vaccines, and the impact of vaccines on cancer incidence will not occur for 15-20 years after implementation. Screening for vaccine-missed cervical cancers will require even more efficient and cost-effective screening tools. Current screening methods, including HPV, are inefficient. Less than a third of women referred for follow-up have preinvasive lesions requiring intervention (CIN 3).

The Early Detection Research Network (EDRN) cervical cancer biorepository (overview diagrammed below) has been developed to provide biospecimens required to support phase 2 and 3 validation studies. Specimens were collected from women attending colposcopy clinics because of abnormal screening results. Colposcopic biopsies are the best end points for disease ascertainment, but inter-observer agreement is relatively poor. The biorepository uses a virtual slide scanning system (Aperio) to archive complete histology data and allow web-based pathology review. These images remain linked to biorepository data, allowing re-review if criteria for diagnosis change. Those enrolled are followed for two years to detect false-negative colposcopic evaluations and allow biomarkers to be monitored in response to therapy. The specimen collection and processing was designed for maximum flexibility, permitting testing of DNA, RNA and protein biomarkers using a wide variety of current and future technologies and assay platforms. Each specimen in the repository has linked clinical, epidemiologic (EDRN common data elements), and HPV typing data. A total enrollment of 2060 women is targeted.

Keywords: cervical cancer screening, biorepository, virtual slide repository



260 DNA Damage Targeted Gene Therapy in Head and Neck Cancer

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TNFerade™ (GenVec, Inc.) is a replication defective adenoviral vector encoding the radio/chemo- inducible DNA sequences (CArG elements) of the Egr-1 promoter ligated upstream of the cDNA encoding human TNF-α. Preclinical data conducted in our laboratory demonstrated increased efficacy of TNFerade™ with radiation and several chemotherapy agents including cisplatin and 5FU in a variety of murine and human tumor models. Our recent studies involve experiments to determine the treatment target. Studies using animals with germ line mutations in TNF receptors I and II (TNFR1-/-, TNFR1,2-/-) and TNF-α (TNF-/-) suggest that a principal target of TNFerade™ is the host stroma which might explain the relatively wide preliminary efficacy of TNFerade™ in varying histological tumor types. In preclinical models we are using MRI to identify regions of the tumor that are most likely viable such that subsequent injections of TNFerade™ are more effective than random injections in a form of adaptive image guidance. In corollary studies we are investigating whether the INF/Stat1 pathway mediates resistance to TNF-α and IR. The Stat1 pathway was characterized in our laboratory using a radio-resistant human head and neck cancer cell line compared with the radiosensitive parental cell line. This Stat1 pathway is of particular interest since it has been correlated with TNF-α resistance *in vitro* and resistance of human breast cancer to chemotherapy and radiotherapy. It is of particular interest that the interferon and TNF-α resistance pathways have recently been shown to intersect.

A phase I dose escalation study of AdGV.EGR.THF.11D (TNFerade™ Biological, GenVec) with concurrent chemotherapy and radiotherapy in patients with recurrent head and neck cancer (HNC) is ongoing. Preliminary findings using the first two dose levels of TNFerade™ ($4x10^9$ and $4x10^{10}$ PU) demonstrate safety with no increase in toxicity profile and two complete responses. Final dose escalation to $4x10^{11}$PU is currently in progress. We are collecting patient biopsies prior to and during treatment. Quantitative measurement of TNF-α encoded by TNFerade™ using qPCR is ongoing. Subsequently patient tissue samples will be evaluated for necrosis/thrombosis by histopathological examination and for Stat1 expression by immunohistochemistry.

Keywords: TNFerade™, head and neck cancer, Phase I trial

261 Evaluation of Redox-Based Non Steroidal Anti-inflammatory Drugs in Models of Cancer Treatment

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An attractive aspect of redox biology involves the ability of small reactive molecules to modulate various processes associated with inflammation and tissue restoration. Applications of non-steroidal anti-inflammatory (NSAID) compounds in the treatment and prevention of cancer such as lung, breast, and colon, as well as demonstrations of increased efficacy of conventional therapies including radiation have led to the development of a novel generation of COX-2 specific NSAIDs as well as NSAIDs modified with redox moieties that release nitric oxide (NO). The latter compounds have the advantage of overcoming side effects such as gut ulceration, heart toxicity, and thrombosis associated with conventional NSAIDs. We have examined the effects of several new NSAIDs that demonstrate antioxidant activities by their abilities to release NO and hydrogen sulfide (H_2S). ACS-2 and ACS-15 are dithiolethione derivatives of valproate and diclofenac respectively, which facilitate mesenchymal to epithelial transitions as well as inhibit cancer cell migration and tumor angiogenesis.

In the non-small cell lung cancer cell (NSCLC) model dithiol thione derivatized valproate (ACS-2) and diclofenac (ACS-15) caused decreased in PGE2 levels and cell proliferation. *In vivo*, both ACS-2 and ASC-15 significantly inhibited A549 and NCI-H1299 tumor growth in nude mice while having no effect on mouse body weight. These results suggest anti-tumor effects without organ toxicity. This tumor growth inhibition may involve upregulation of E-cadherin expression. Moreover, NSCLC *in vivo* growth inhibition by gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was increased in the presence of ACS-2. These results suggest that the ACS NSAIDs anti-tumor effects may involve increased E-cadherin expression, facilitating the mesenchymal to epithelial transition of NSCLC cells. Preliminary results also show that ACS derivatized complexes increase E-cadherin protein levels in MB-231 breast carcinoma cells, which correlated with inhibition of cell migration. These derivatized NSAIDs have favorable properties, which can increase the efficacy of some treatment modalities and targeted mechanisms involved in metastatic disease.

Keywords: oxidative, inflammation, NSAID

262 Inhibition of Lung Carcinogenesis by Tea Polyphenols and Atorvastatin

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The objective of this project is to study the inhibition of lung carcinogenesis by green tea polyphenols and the synergistic action when used in combination with atorvastatin (ATST, trade name Lipitor). Tea is commonly consumed by humans, and Lipitor is a popular cholesterol-lowering drug. Our hypothesis is that green tea polyphenols, especially the major polyphenol epigallocatechin-3-gallate (EGCG), interact synergistically with ATST in the inhibition of lung carcinogenesis. EGCG is known to suppress cell proliferation, enhance apoptosis, and inhibit angiogenesis through modulation of signal transduction pathways. ATST is known to inhibit HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase and the biosynthesis of isoprenoids and cholesterol, and is hypothesized to influence membrane association of small G-proteins. The interaction of the different types of mechanisms may generate synergistic actions in the inhibition of carcinogenesis. The possible use of the combination of EGCG and ATST for lung cancer prevention is an attractive strategy that needs more investigation. To test our hypothesis, these agents will be studied alone and in combination in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung carcinogenesis model in A/J mice and related cell lines. The inhibitory action and the mechanisms involved will be thoroughly investigated with specific aims as follows: 1) Determine the inhibitory actions of EGCG and its combination with ATST in an NNK-induced mouse in lung carcinogenesis model at the post-initiation and progression stages. Possible synergistic action and levels of EGCG and ATST will be analyzed. 2) Elucidate the mechanisms of inhibition of lung carcinogenesis by EGCG and its combination with ATST in NNK-treated mice. Short-term animal experiments with adenoma and adenomcaricnoma-bearing mice will be used as a direct approach to obtain mechanistic information *in vivo*. 3) Delineate detailed mechanisms of lung cancer prevention by EGCG and its combination with ATST in lung cancer cell lines. We will integrate the results from studies *in vitro* and *in vivo* to gain a better understanding of lung cancer preventive activities of EGCG (and tea polyphenols) and its combination with ATST. (Work supported by NIH grant CA 133021)

Keywords: prevention, lung cancer, EGCG and atorvastatin

263 Daidzein-Metabolizing Phenotypes Among Premenopausal Women in the United States: Relationship to Health and Lifestyle Factors and Impact on Breast Density and Sex Steroid Measures

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Greater exposure to estrogen throughout a woman's lifetime increases her risk of developing breast cancer. In the gut, the microbiota play a substantial role in estrogen metabolism; therefore, inter-individual differences in host bacterial populations may be a determinant of estrogen exposure and ultimately of breast cancer risk. Production of equol from the isoflavone daidzein by gut bacteria serves as a biomarker of unique intestinal bacterial populations. A third to half of individuals have the gut bacteria capable of converting daidzein to equol. Evidence from several studies suggests that, irrespective of soy intake, women with the capacity to produce equol have hormonal profiles associated with lower risk of breast cancer. Equol-producer phenotypes can be determined readily from urine collected after a 3-d soy challenge.

We examined relationships between equol-producer status and breast density, steroid hormones, and markers of estrogen metabolism [2-hydroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OHE1)]. We also evaluated relationships between daidzein-metabolizing phenotypes and demographic, anthropometric, lifestyle, and dietary factors. We recruited healthy female volunteers (40-45 y) from within the Group Health system, who had had a screening mammogram in the past year. 200 women provided a first-void urine sample after a 3-d soy challenge, and completed a health and demographics, physical activity, and food frequency questionnaires, and a 3-d food record. They provided fasting blood and spot urine samples during d 5-9 of their menstrual cycle. Soy-challenge urines were analyzed for isoflavones to determine equol-producer phenotypes; serum was analyzed for estrogens, androgens, and SHBG; spot urines were analyzed for 2-OHE1 and 16α-OHE1. Percent density on recent (<14 months prior to their clinic visit) mammograms was assessed by one reader using a computer-assisted method. Multiple regression analysis was used to assess relationships between equol production and breast density and the hormone measures.

Fifty-five women (27.5%) had detectable urinary equol and were classed as equol producers. Compared to non-producers, equol-producers were more likely ($p \leq 0.05$) to be Hispanic or Latino, highly educated, and to have frequent constipation. Equol-producers reported higher overall physical activity than non-producers. Data were log-transformed and multiple regression analyses were conducted to assess relationships between daidzein-metabolizing phenotypes and hormones and SHBG. Data from 187 and 189 women were included in analyses of serum and urine hormones, respectively. In unadjusted analyses, equol-producers ($n = 52$) had lower free testosterone than equol non-producers ($n = 137$, $p = 0.02$); however, in adjusted analyses, this difference was no longer significant. Similarly, in both unadjusted and adjusted analyses, there were no differences in breast density between equol producers and nonproducers ($p > 0.05$). Our findings suggest that in the absence of regular soy exposure, there is no effect of the equol-producer phenotype on sex-steroid-related biomarkers in premenopausal women.

Keywords: breast density, equol, steroid hormones

264 Disparity Between Serum and Nipple Aspirate Fluid (NAF) Estradiol Concentrations During the Menstrual Cycle

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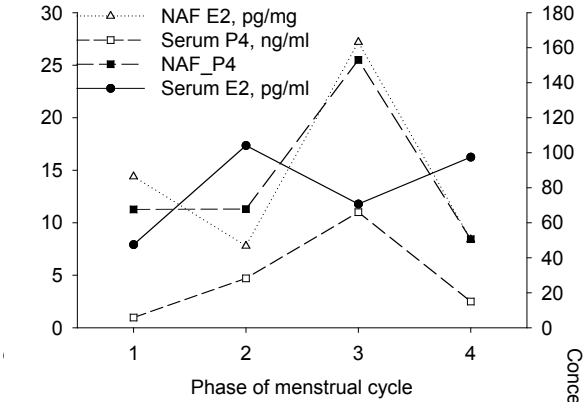
Background: NAF, elicited from healthy women, may provide information about the hormonal environment of the breast and the potential risk for breast cancer. We have studied NAF sex hormone changes across the menstrual cycle and after menopause to determine the relationship to serum concentrations.

Methods: Subjects were women attending the Lynn Sage Breast Center at Northwestern Memorial Hospital. Samples of NAF from 20 premenopausal and 23 postmenopausal women were evaluated. Premenopausal women were divided into groups 1 through 4, backdating from the day of menstruation, -28 through -19, -18 through -13, -12 through -6 and -5 through 0, respectively. NAF and venous blood were collected using standard methods. Total protein was measured by a fluorometric procedure. Unconjugated steroids, estradiol (E2), estrone, progesterone (P), and testosterone (T), were extracted into ethyl acetate-hexane (3:2) and were separated by HPLC on a C-18 column with gradient elution before assay. DHEA sulfate, estrone sulfate, and cathepsin D were measured in the aqueous fraction. All analytes were measured by immunoassays. Transformation of the data of all analytes to their natural logarithms normalized the data. Data were analyzed by ANOVA.

Results: The protein concentration in the samples was similar among all groups, mean = 128 mg/ml (P=0.904). In NAF only E2 and P differed during the menstrual cycle, P = 0.064 and 0.007, respectively, but the pattern of NAF E2 followed that of NAF P and not serum E2. The figure shows the geometric mean values at each phase of the menstrual cycle. The estimated time of ovulation is shown by the vertical line. With the exception of P and DHEA sulfate, which were lower, analytes in samples from postmenopausal women were not significantly different from those from premenopausal women.



Comment: The most significant finding is that NAF E2 does not parallel serum levels but follows closely NAF P levels. NAF E2 was actually lowest when serum E2 was highest. Changes in other analytes during the menstrual cycle were minimal. Estradiol, its direct precursors, and the E2-related protein, cathepsin D, were not decreased in NAF after menopause.

Keywords: estradiol, nipple fluid, breast cancer risk

265 Development of an RNA Sensor Platform for Detection of Circulating Tumor Cells

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We are developing a chip-based RNA sensor, with an initial application for detection of circulating tumor cells. Circulating tumor cells are harvested from blood using a simple porous membrane gradient centrifugation device, and RNA is extracted. Target RNAs from specific cancers are selected from the literature, and library selection is performed to optimize antisense oligonucleotide (ASO) binding sites. Detection is based on a "hybridization sandwich", with one ASO_1 covalently attached to silicon or rhodium nanowires, and the other ASO_2 covalently attached to a 40nm Au particle. Formation of the ASO_1-RNA-ASO_2 sandwich induces a shift in the resonance frequency of a nanowire cantilever, which is measured optically. We have optimized ASO sites for a number of target RNAs for prostate, breast, and melanoma cancers, developed conditions that allow single nucleotide mismatch discrimination, shown that the ASO-derivatized nanowires remain functional following assembly and on-chip integration, developed the ability to assemble derivatized nanowires at predetermined chip locations with high yield, and shown that femtogam-level formation of each target RNA sandwich complexes induce a shift in resonance frequency that can be easily detected. The platform is currently being extended to allow quantification of proteins, by replacing the ASOs with aptamers, and the next phase of development will include transitioning to direct electrical readout of binding events. We have initiated clinical trials with melanoma patients; samples will be analyzed using conventional techniques (QPCR, ELISA) for benchmarking, and the balance of the samples will be analyzed using the RNA sensor platform when it is available.

This work is supported by grant CA118591 from the NIH/NCI IMAT Program.

Keywords: sensor, nanotechnology, CTCs

266 Automated Rapid Analysis of Surgical Margins of Breast

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Multiple randomized prospective trials with greater than ten year follow-up have proven that breast conservation therapy (BCT) has equal survival efficacy compared to mastectomy in treating early stage breast cancer and for this reason currently BCT has become the standard of care to treat this malignancy. However, obtaining a negative margin in localized excision with primary BCT is still a challenge. Many studies show that local recurrence is significantly higher in patients with a positive margin versus a negative margin excision. Despite improved pre-operative imaging techniques, such as breast MRI and ultrasound, many studies report positive margin rates of 25-50% for partial mastectomy, even in early stage breast cancer patients.

A method has been developed to detect cancer cells in breast tumor surgical margins. Traditionally, breast cancer is diagnosed in tissue samples by architectural features of the tissue including an increased number of epithelial cells growing within or outside the mammary ducts. Unfortunately, breast cancers are heterogenous and there are no reliable cancer marker for detecting breast cancers cells especially when evaluating limited numbers of cells. We performed a clinical study of surgical margins sampled by touch imprint cytology and have shown that the presence of cancer can be reliably determined from the density of epithelial cells captured onto the imprint slides. A rapid staining procedure for epithelial cells and an automated microscopy algorithm for counting single and clustered epithelial cells were developed. We determined that the density of epithelial cells harvested in imprint cytology of surgical margins could be employed to rapidly evaluate the surgical margins without disturbing the tumor. This technique is both sufficiently rapid for intraoperative use and preserves the tumor for permanent section pathology thereby rendering the new technique compatible with current surgical practice. Our study has shown that the technique is very accurate for margin evaluation for invasive ductal carcinoma (IDC). However, margin evaluation for ductal carcinoma insitu (DCIS) may require additional automated microscopy and analysis of the shape and staining of individual epithelial cell nuclei. The ability to procure freshly harvested breast cancer cells also permits “panning” and evaluation of novel cancer markers . In the future we plan to utilize quantum dot labeled nanoparticle markers to identify novel markers on the surface of breast cancer cells. Nanoparticles are quite attractive since multiple markers can be developed and simultaneously used to overcome the typically modest differences in protein expression between normal epithelial cells and low grade cancer cells. For example, with six distinct nanoparticle markers, protein expression of just 2 between normal and cancer cells will be sufficient to distinguish cancer from non-cancer cell clusters with 95+% certainty.

Keywords: surgical margins, automated microscopy, breast cancer

267 Breast Cancer Risk Associated With BRCA1 and BRCA2 Variants of Uncertain Significance

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Interpretation of results from mutation screening of the breast and ovarian cancer predisposition genes *BRCA1* and *BRCA2* is a common part of clinical practice. In most cases, this is straightforward, but when rare variants of uncertain significance (rare missense and intronic variants) with an unknown influence on protein function and an undefined contribution to cancer development are identified, problems in interpretation are encountered. Variants of uncertain significance account for approximately 37% of all known variants detected by clinical mutation screening in *BRCA1* and *BRCA2* and are a significant clinical genetics issue. Clinical utility of BRCA testing would be greatly increased if these variants could be reliably classified in terms of their predisposition to disease.

In order to better assess the clinical significance of *BRCA1* and BRCA2 variants identified through genetic testing we used a combination of genetic and functional studies. The genetic studies involved determination of the odds of cancer causality of each mutation using a combined likelihood model encompassing information on co-occurrence of variants with other known deleterious mutations, detailed analysis of personal and family history of cancer in carriers of variants and analysis of co-segregation of variants with disease in pedigrees. Genetic data were derived from the Myriad Genetics Laboratories database of nearly 70,000 full-sequence tests and additional families ascertained as part of an ongoing study of breast cancer families carrying missense *BRCA1* and *BRCA2* mutations at the Mayo Clinic. A total of 133 *BRCA1* and *BRCA2* variants had odds of at least 100:1 in favor of neutrality whereas 12 had odds of 1000:1 in favor of being deleterious and almost certainly predispose to breast and ovarian cancer. Another 43 had odds of at least 20:1 in favor of being deleterious. Of these, three *BRCA2* and five *BRCA1* variants were defined as cancer causing because they promote aberrant RNA splicing. As the genetic approach to classification is limited by availability of family data, we further characterized a series of *BRCA2* missense mutations using *in vitro* functional assays that measure the ability of wildtype and mutant forms of *BRCA2* to repair DNA damage in cells by homologous recombination and to regulate the centrosome numbers in cells. Eight missense mutations classified as deleterious using family data disrupted homologous recombination repair whereas seven failed to control centrosome amplification. Likewise eight known neutral variants displayed wildtype BRCA2 activity in both assays. Sensitivity was estimated at 100% for the homologous recombination assay and 85% for the centrosome amplification assay. Specificity was approximately 100% for both assays.

In summary, the strong correlation between the functional assays and the genetic studies suggest that it may be possible to classify variants in *BRCA2* using functional assays alone in the absence of genetic data. These results will prove useful for improved risk assessment of carriers of these *BRCA2* variants and their family members and for validation of other approaches to mutation classification. The validation of the functional assays also has important implications for classification of the many variants in *BRCA2* that have been found in small numbers of families.

Keywords: BRCA1, BRCA2, variants of uncertain significance

268 Gene Signature of Cancer Stem Cells is Manifested Within an Intrinsic Subgroup of Breast Cancers With Mesenchymal Properties

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Background: Breast cancer stem cells characterized by $CD44^{+}/CD24^{-/low}$ may be resistant to therapy and responsible for relapse. Mammospheres (MSs) can be propogated as an in vitro surrogate assay for increased self-renewal. We set out to define a "signature" expression pattern ssociated with $CD44^{+}/CD24^{-/low}$, mammosphere-forming cells.

Methods: Breast cancer biopsies (n=19) were digested, stained with CD24, CD44, and lineage antibodies, and analyzed by flow cytometry. A portion of the unsorted cells were plated under serum-free conditions to form MSs (n=16). Gene expression, using the Affymetrix U133 GeneChip platform, of cancer cells bearing $CD44^{+}/CD24^{-/low}$ *vs.* other sorted cells, and between cancer MS *vs*. the primary invasive cancers were analyzed. Gene expression from two trials (neoadjuvant letrozole N=18, and docetaxel, N=12) were used as validation studies.

Results: In the flow-sorted $CD44^{+}/CD24^{-/low}$ *vs.* other cells, 1,424 named genes were elevated ($p<0.01$, fold change>1.5, FDR=0.20). The comparison between MSs *vs.* primary cancers yielded 1,890 elevated genes (FDR=0.25). Between the two sets, 380 genes were in common, a highly significant overlap (p=1E-5, one-sided Fisher's exact). This overlap was ~40% greater than what would be expected (n=110) if the two sets had no biological relevance. Differential pathways included genes in PI3K/AKT signaling (PI3K3R3, ErbB3, FGFR2, PRLR), and the Notch pathway—a known regulator of normal and malignant stem/progenitor cells (Jagged-2, MAML2, Deltex, HES1). This signature was found exclusively activated in tumors of the recently identified "claudin-low" subtype, characterized by overexpression of many mesenchymal genes. Both signatures were validated in two independent data sets comparing the expression profiles of paired breast cancer core biopsies before vs. after letrozole or docetaxel chemotherapy.

Conclusion: The mesenchymal association provides a possible explanation for the intrinsic resistance of breast cancer stem cells to therapy.

Keywords: cancer stem cells, epithelial mesenchymal transition, therapy resistance

269 *In Vitro* Systems Approaches to Identification of Molecular Markers that Predict Clinical Response in Breast Cancer

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Opportunity: The pharmaceutical industry estimates that over 400 drugs are now in phase II/III clinical trials for diverse cancer indications. Early development of biomarkers that predict clinical response will allow these drugs to be tested in tumors most likely to respond. This will increase the probability that drugs effective in tumor subpopulations will not be missed and will decrease the cost of early trials by allowing them to be tested in small numbers of patients whose tumors are most likely to respond. It also will allow predictive biomarkers to be developed in parallel with experimental drugs so that successful drugs enter the clinical market with fully validated predictive biomarkers.

Background: Genome scale analyses have established that cancer cells manifest a variety of genomic, transcriptional and translational features that affect disease pathophysiology and response to therapy. Assays for these discriminatory molecular features are in use as guides to disease management. In breast cancer, for example, molecular predictors of outcome are now in routine use to identify patients at low risk of progression so they can be spared unnecessary therapy while assays for ERBB2 amplification or over expression are being used to select tumors for treatment with trastuzumab or lapatinib. A fast and cost-effective approach to predictive marker identification is to use sufficienlty large collections of well-characterized cancer cell lines that accurately model the biological, genetic and epigenetic diversity of the disease to develop markers to guide clinical trials. The general strategy involves measuring responses to treatment by cells whose molecular characteristics are known. These datasets are then analyzed to identify molecular features that are significantly associated with response. *In vitro* approaches clearly reveal ERBB2 amplification as a predictor of response to trastuzumab and lapatinib and EGFR mutation as a predictor of response to gefitinib. These successes motivated our efforts to use an *in vitro* system comprised of 50 breast cancer cell lines to identify molecular features associated with pathway targeted drugs that are now being developed and to refine predictors of approved drugs that are already in use. This *in vitro* systems approach is appealing since candidate predictive markers can be discovered sufficiently early so that markers and drugs can be co-developed during early phase clinical trials.

Implementation: In order to stringently test the in vitro approach, we have developed novel computational and experimental approaches for identification of non-linear signatures of response and we have applied these in assessing responses to ~40 approved and experimental therapeutic agents. We have tested the clinical utility of one of these; a 6-gene predictive assay for lapatinib developed using this approach, in two independent clinical trials. We demonstrate that the *in vitro* predictor does improve the ability to predict response to lapatinib even in ERBB2 positive patients.

Keywords: predictive biomarkers, lapatinib, systems biology

270 Host Polymorphisms in the IL6 Promoter Predict Poor Outcome in Patients With ER-Positive, Node-Positive Breast Cancer

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Interleukin-6 (IL-6) modulates immune response, estrogen production and growth pathways in breast cancer. Common polymorphisms in the *IL-6* promoter regulate transcription. We previously reported an association between the IL6-174GG genotype and decreased disease-free and overall survival among women with ER-positive, node-positive breast cancer[1]. We sought to further evaluate the full complement of functional variants in the IL6 promoter (SNPs -572G>C, -597G>A and repeat -373 A_nT_n) in node-positive patients enrolled on a multicenter, cooperative group trial of adjuvant chemotherapy for breast cancer.

Genomic DNA and clinical data from patients enrolled on E2190/Intergroup 0121 were collected. Genotyping for -597G>A, -572G>C, -174G>C and -373AnTn was performed by PCR using site-specific primers and PyroSequencing and sequencing for the repeat polymorphism. Log-rank tests and Cox modeling were used to compare outcomes by genotype/haplotype and other factors. Stratification on estrogen receptor status was prespecified.

344 patients (64% of parent trial) had corresponding genotype/clinical data available and did not differ from overall trial participants. After adjustment for other prognostic factors, those patients with ER positive tumors and genotypes 597GG or 174GG had significantly worse DFS (HR 1.6, p=0.02 and HR 1.71, p=0.007, respectively), while the 373 8A12T repeat appeared to be protective (HR 0.62, p=0.02). The G-G-[10A/11T]-G haplotype was associated with worse DFS (HR 1.46, p=0.04), while the A-G-[8A/12T]-C haplotype was associated with improved DFS (HR 0.69, p=0.009).

Functional polymorphisms in *IL-6* identify an ER-positive population with poor outcome. These findings support mechanistic studies aimed at development of host-targeted approaches to improve treatment for ER-positive breast cancer.

Keywords: breast cancer, polymorphism, Interleukin-6

271 Effect of Protein Phosphatase 2A Subunit Gene Haplotypes and Proliferative Breast Disease on Breast Cancer Risk

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A large number of genes encode components of complex growth factor signaling networks and may contribute to breast cancer risk. Prominent among these candidates are protein phosphatases, countering kinases of signaling cascades. We explored the role of a set of candidate genes encoding subunits of the major cellular protein phosphatase 2A (PP2A) in a cohort of women with a history of benign breast disease. Women with a history of benign breast disease are at increased risk of subsequently developing breast cancer.

We evaluated genetic variation of a set of 49 candidate genes of signal transduction pathways for potential contribution to breast cancer risk. The study was conducted as a nested case-control investigation of the cohort. DNA prepared from the original archival FFPE benign breast biopsy was available for 301 women diagnosed with breast cancer on follow-up, and for 545 of their 602 matched controls. Controls were women who remained free of breast cancer, matched to cases in a 2:1 ratio by race, age, and year of entry biopsy.

We observed that a PP2A structural subunit gene, *PPP2R1A*, and two regulatory subunit genes, *PPP2R2A* and *PPP2R5E*, each appeared to modify breast cancer risk with both risk-elevating and risk-reducing genetic variants. Only *PPP2R2A* was significantly associated with breast cancer after correction for multiple comparisons, with significant risk and protective haplotypes (OR=1.79, study-wide P=0.015, and OR=0.62, study-wide P=0.025, respectively). These variants interact with a history of proliferative breast disease to modify risk for breast cancer. In a dose model that included all three genes, breast cancer risk ranged from a 5.1-fold decreased to a 3.9-fold increased risk, according to the portfolio of variants inherited by a woman (study-wide P=0.02).

If confirmed by independent studies, PP2A may play a key role as a tumor suppressor in breast cancer, able to augment as well as to nullify elevated risk associated with early benign proliferative breast disease.

Keywords: breast, formalin fixed paraffin embedded, proliferative

272 Development of the "PAM50" Intrinsic Subtype Assay: Potential as a Prognostic Assay for Patients With Early Stage Breast Cancer and as a Predictive Assay for Patients Undergoing Neoadjuvant Aromatase Inhibitor Therapy for ER+ Disease

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Background: We have shown by microarray and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) that breast tumors can be reproducibly classified into five distinct groups based on the expression profile of a "minimal" intrinsic gene list henceforth called the PAM50. The PAM50 identifies two ER+ subgroups - Luminal A (LumA) and Luminal B (LumB), two ER- subgroups - HER2-enriched (HER2) and Basal-like, and a final category Normal-like which has an expression pattern that most closely resembles non-malignant breast tissue. This biologic classification has been shown to be an independent predictor of disease free survival when considering standard clinical parameters. However, risk predictors based on these gene expression profiles have been difficult to clinically implement. We sought an objective method to predict subtypes of breast cancer and generate a continuous risk score based on a biological subtype predictor that can be performed on archived tissue blocks using real-time qRT-PCR.

Methods: Microarray and real-time qRT-PCR data from 189 samples, procured as fresh frozen and formalin-fixed paraffin-embedded tissues, were used to statistically select prototypes for the biological subtypes of breast cancer. Classification algorithms were developed using four independent breast microarray studies together comprising 1244 cases. From these data, a risk of recurrence (ROR) predictor was developed based on distance to the PAM50 subtype centroids. In addition, a proliferation score was generated from the expression of 11 tightly correlated, cell cycle-regulated genes in the PAM50 to create a tailored ROR score for response to neoadjuvant aromatase inhibitor therapy. A linear relationship between the ROR score and relapse risk was identified across the cohorts.

Results: The biological subtypes predicted on the combined microarray test sets showed prognostic significance in all stages of disease (1244 subjects; p=7.1e-14), node negative disease with no adjuvant systemic therapy (733 subjects; p=6.2e-7), and in patients treated with endocrine therapy (404 subjects; p=1.3e-3). The proliferation weighted ROR model applied to tumor expression profiles generated from baseline biopsy, one month neoadjuvant treatment, and subsequent tumor excision at surgery showed marked changes with aromatase inhibitor treatment, and robustly identified a group of resistant tumors on the basis of a persistently high risk of relapse score one month after treatment. These resistant tumors were associated with a poor clinical response rate, poor pathologic response in the surgical specimen, and high relapse rate in follow-up.

Conclusion: A qRT-PCR panel of 50 discriminator genes, applicable to standard pathology blocks from biopsy and excision specimens, robustly identifies the intrinsic biological subtypes of breast cancer and predicts response to aromatase inhibitor therapy as well as overall prognosis in independent series.

Keywords: quantitative RT-PCR, bioclassifier, aromatase inhibitor

273 Inhibition of Aromatase for Breast Cancer Prevention in Postmenopausal Women on Hormone Replacement Therapy

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Conversion of androgens to estrogens is controlled by the enzyme aromatase. In the postmenopausal woman aromatase activity in the breast will provide the major source of local estrogen. Not only is up-regulation of aromatase observed in areas of benign breast disease, but aromatase inhibitors have been shown to be more effective than tamoxifen for preventing contra-lateral breast cancers in postmenopausal women. Pre-clinical data in aromatase over-expressing animals suggest that the aromatase inhibitor, letrozole, is effective in a high estrogen environment.

We hypothesized that administration of letrozole would inhibit progression toward breast cancer in postmenopausal women who were taking systemic hormone replacement therapy (HRT) for relief of menopausal symptoms. We reasoned that letrozole would result in a reduction in breast epithelial cell proliferation, which would serve as a surrogate endpoint biomarker of response. Further, we anticipated that the combination of letrozole and HRT would not diminish the relief provided by HRT; and that the low dose estrogen might actually prevent some of the side effects that have been associated with aromatase inhibitors.

In a pilot study funded by Novartis Pharmaceuticals Corporation, we recruited postmenopausal women at high risk for development of breast cancer and taking a stable dose of estrogen or estrogen plus progestin, who were then screened by random periareolar fine needle aspiration (RPFNA) of the breast. To be eligible, the acquired breast epithelial cells had to be characterized as cytologic hyperplasia plus atypia or borderline atypia, with expression of the proliferation marker Ki-67 by immunocytochemistry. Forty-two women were enrolled in the one arm study and received 2.5 mg letrozole per day for 6 months, followed by repeat assessment of biomarkers. Ki-67 was reduced by a median relative value of 66%. There was no significant change in breast cell cytomorphology; ER weighted index score; serum estradiol, testosterone, or IGF-1:IGFBP-3 ratio; mammographic breast density, or frequency or severity of perimenopausal symptoms [Breast Cancer Res Treat 2007; 106:75-84].

A randomized, double-blind Phase II trial is now underway in which high risk postmenopausal women on HRT will be screened by RPFNA. Women with specimens exhibiting cytologic hyperplasia with or without atypia plus Ki-67 expression $\geq$1.5% are eligible and randomized to receive letrozole 2.5 mg daily or matched placebo for six months. The primary endpoint is reduction of proliferation (Ki-67) at six months; secondary endpoints include change in breast tissue estradiol levels, nuclear morphometry, and expression of estrogen responsive genes by qRT-PCR. All women may then choose to receive open-label letrozole for the next six months, followed by another RPFNA. To achieve the target goal of 108 subjects, six collaborating institutions will accrue subjects. The trial is funded jointly by the NCI Division of Cancer Prevention (RO1 CA122577) and Novartis, which is also providing the study agents.

Keywords: random periareolar fine needle aspiration (RPFNA), breast cancer, chemoprevention

274 Hypermethylated Gene Markers: Pilot Studies Testing Their Utility in Risk Assessment and as Biomarkers of Cancer in Women With Spontaneous Nipple Discharge

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Gene promoter hypermethylation is a hallmark of breast cancer. We recently developed an assay called quantitative multiplex-methylation-specific PCR (QM-MSP) that, in the ductal lavage setting, detected breast cancer cells in cytological fluids with over twice the sensitivity of cytology alone. The QM-MSP assay can evaluate a panel of up to 12 genes in patient samples containing as few as 500-1000 cells. We have recently tested its utility in two pilot studies-first, to enhance detection of cancer in ducts with spontaneous nipple discharge (SND) and second, as a risk assessment marker in the contralateral (CLB) breast of women being treated with anastrozole.

In the first study, ductoscopy was performed in the course of evaluating women with SND; ducts with significant ductoscopic abnormalities were surgically resected (36 ducts in 33 women) and those with minimal findings were not (28 ducts in 16 women). Gene promoter hypermethylation levels were assessed by QM-MSP for 11 genes in cells harvested during ductoscopy and results were compared to ductoscopy findings, and tissue histology/cytology. Higher levels of methylation occurred in cells from ducts with malignant lesions compared to those with benign lesions such as papilloma ($p = 0.006$); or ductoscopically normal ducts ($p = 0.0001$). Cumulative *RASSF1A, TWIST1,* and *HIN1* gene methylation accurately distinguished ducts with cancerous vs. benign lesions (78% classification accuracy, 100% specificity and 72% sensitivity according to ROC analyses). QM-MSP analysis was more informative then cytology (100% vs. 29% sensitivity, respectively), for detecting cancerous cells. The positive predictive value of ductoscopy more than doubled (19% vs. 47%) by adding QM-MSP to ductoscopy, demonstrating the benefit of targeting ducts having both high methylation and significant ductoscopic abnormalities for surgical excision. Future large-scale studies are needed to validate this approach.

Women with a history of breast cancer are at increased risk to develop a contralateral breast (CLB) cancer. Since methylation is frequently detected in normal tissues adjacent to a breast tumor, we hypothesized that methylated genes would be detected in CLB of women with prior breast cancer and that treatment with anastrozole, an aromatase inhibitor that reduces the risk of CLB cancer, would decrease gene promoter methylation. We conducted a prospective, single-arm study in 54 postmenopausal women with hormone receptor-positive stage 0-III breast cancer who had completed local therapy, had an intact CLB, and would receive anastrozole as their sole adjuvant therapy. Of those, 33 women underwent an optional CLB biopsy both at baseline and 6 months after initiating anastrozole. At baseline, 84% of paired samples had measurable cumulative methylation of the 6 gene panel; Twist (33%), RASSF1a (28%), and RAR-beta (34%) were most frequently methylated. After 6 months of anastrozole, we observed significant decreases in methylation for Twist ($p=0.004$), RASSF1a ($p=0.008$), and RAR-beta ($p=0.03$), among patients with methylation identified at baseline. These results suggest that methylation markers hold promise for risk assessment, and warrant larger studies involving prospective evaluation of the relationship between changes in methylation in response to preventive interventions and incidence of breast cancer in high-risk women.

In summary, QM-MSP based evaluation of breast tissue/cells has the potential to provide a valuable assay for risk assessment and early detection of breast cancer.

Keywords: breast cancer, methylated gene markers, risk

275 The NCI-Cooperative Breast Cancer Tissue Resource (CBCTR)

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Biospecimens are critical to translational research, particularly in the area of prognostic and predictive markers. Specimens must be of high quality when first obtained and when prepared for other investigators, often many years later. By themselves such specimens are of limited use but become increasingly valuable as the period of follow-up increases and as layers of annotations are added to initial clinical data. The NCI addressed the need of laboratory investigators for large numbers of well-characterized and annotated specimens for definitive evaluations of prognostic markers by creating the CBCTR.

In 1994-96 the CBCTR collected over 9200 specimens of invasive and non-invasive breast cancer from the four institutions listed above, each site contributing about ¼ of the total. All cases were reviewed by study pathologists according to a shared Pathology Manual. A common set of clinical and follow-up data were gathered from tumor registries and other local sources, encoded and placed into a single database managed centrally by Information Management Services (IMS). A comprehensive Manual of Operations (MOO) governed all data acquisition and entry. This MOO has been frequently amended and updated to keep up with new developments in breast cancer biology and in response to investigator comments and requests. To protect confidentiality of the specimens and to meet HIPAA requirements, all data are deidentified before they leave the institution.

Breast cancer tissue material is available in two forms: sections of individual blocks from a case or group of cases meeting a set of criteria (e.g. over age 50, positive nodes, ductal histology, 10-years of follow-up) or in already-constructed tissue microarrays (TMAs). Currently, we are distributing two TMAs: a Progression Array of 288 breast cancers of all stages, normal breast tissue controls, cell lines and a Prognostic Array of about 500 Stage 1 breast cancers and similar controls. A Stage 2 Prognostic Array is being made and will be available at the end of 2008. A unique feature of the CBCTR TMAs is their careful design and oversight by NCI statisticians to assure laboratory investigators of statistically meaningful results with their use.

Investigators interested in tissue sections may search the online CBCTR database to determine if adequate numbers of cases exist to meet their needs. Letters of Intent and full Applications from all requestors are reviewed by a Research Evaluation Panel (REP) which reviews the science of the proposed study and submits its recommendations to the Coordinating Committee of the CBCTR which, in turn, decides whether to provide tissues.

The CBCTR was originally developed for definitive evaluations of markers of prognosis. It has been used increasingly for initial studies of newly discovered markers at relatively early stages of development. The cases come from widely different sites and, in toto, represent a reasonable cross-section of breast cancers that occur in the community. As such, patients have been treated in the community by widely divergent methods but not usually on protocol studies. This resource seems well-suited to studies of breast cancer biology and perhaps for investigations of the interaction of markers with particular therapies.

Keywords: cancer tissues, breast cancer, resources

276 Biomarkers to Predict Subsequent Tumor Events in Women Diagnosed With DCIS

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Opportunity: Ductal carcinoma *in situ* (DCIS) has become a relatively common diagnosis, yet the clinical and biologic significance of DCIS lesions is not fully understood. It appears that 5 to 10% of DCIS cases are associated with a subsequent invasive cancer within 5 years and a similar proportion has a subsequent DCIS lesion. Clinical trials and population-based studies have failed to consistently identify characteristics of women who are diagnosed with DCIS who have a high or low risk of subsequent invasive cancer. Identifying molecular marker signatures that can accurately predict subsequent DCIS and/or invasive cancer could aid in stratifying an individual's risk for subsequent tumors after a DCIS diagnosis and allow women to better tailor treatment choices to avoid unnecessary adjuvant therapy and opt for aggressive treatment when needed.

Background: The first molecular portraits of breast tumors were generated using microarray analysis. These studies individualized tumors into several subclasses of luminal-like and basal-like categories that continue to evolve in sub-classification. While several molecular markers from these studies are being used to predict invasive tumors with poor prognosis or response to therapy, this information is not yet being applied to risk stratification of DCIS. One effective approach to marker identification for early stage disease has been to examine the earliest changes in epithelial cells using *in vitro* primary explants of human mammary epithelial cells. The general strategy involves the growth and characterization of the human breast epithelial cells as they acquire malignant phenotypes. This approach has lead to the discovery that a compromised pRb pathway leads to epigenetic remodeling and the generation of aneuploidy. Additionally, the application of *in vitro* results also has shown that DCIS samples containing proliferating cells identified using the Ki67 proliferation marker combined with the over expression of p16 and/or COX-2 proteins reflects abnormal response to cellular stress and predicts subsequent tumor events (Gauthier et al, Cancer Cell 12, 479-91, 2007). This suggests that when breast tissue shows stress activation and deregulation of p16 and Rb signaling it expresses a defining signature of basal-like carcinogenesis that can be assayed before the development of invasive disease.

Implementation: In order to validate *in vitro* and pilot study results, we have developed a population-based cohort of women with DCIS diagnosed in Northern California and collected tissue samples to test biomarker signatures. We have demonstrated that (a) novel biomarkers can be developed in an *in vitro* system and applied to identify women at high risk of subsequent invasive tumors suggesting an *in vitro* approach can be used to further develop biomarker profiles to identify women at low and high risk of subsequent tumors after a DCIS diagnosis. We have also shown that (b) these markers can be validated in a cohort of a relevant population. Since basal-like breast carcinomas comprise 39% of disease in young African American women, this would be a relevant population to test. Finally, to bring such a prognostic test into full use, (c) the signatures identified should be validated in a prospective cohort.

Keywords: prognostic signatures, risk stratification, DCIS

277 Biomarkers for Caloric Restriction in Rats: Biomarkers for Cancer Risk in Humans?

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Caloric restriction (CR) in rodents is associated with decreased morbidity and increased longevity in rats -- a complement to the observation that obesity is associated with increased morbidity and mortality in humans. The phenotypic effects of CR are remarkably robust. As one example of the power of the CR paradigm, rates of mammary cancer incidence are generally reduced at least 90% in CR animals, fewer tumors are found in these animals, and those tumors found are reduced in size. CR appears to affect initiation, promotion, and progression phases of the disease, and may be dominant against genetic predisposition, specific components of the diet, and specific environmental carcinogens.

We therefore propose that biomarkers that can identify *ad libitum* fed and CR rats with a high degree of accuracy will predict disease risk in humans (ie, by, in part, recognizing a metabolism reflective of resistance to disease). One complication is that the metabolic state characteristic of caloric intake might encompass at least four elements: (i) short term response to lower food intake; (ii) body weight/body mass index; (iii) long-term adaptation to low calorie diets, and; (iv) beneficial physiological effects associated with the response to such diets. The "signal" of such a response may be further confounded by effects across different tissues, times and, in outbred populations, eg humans, in the complex gene/environment (eg diet) interactions.

Omics-based approaches offer a possible means around the limitations induced by complexity. The strategies focus on melding analytical technologies capable of simultaneously querying multiple biochemical compounds/pathways etc with computational approaches and workflows capable of finding signal in a large background of noise. Metabolomics and proteomics are well suited to the types of ongoing epidemiological studies.

We have therefore developed serum metabolomic profiles that can identify *ad libitum* fed and caloric-restricted rats with a high degree of accuracy. These profiles have been adapted for use in human epidemiology studies, with several long-term goals, including objective analysis of diet in humans and individualized risk prediction for diseases involving metabolic components, such as breast cancer. Exploratory studies previously identified 93 redox-active small molecules from sera (measured by HPLC coupled with coulometric detector arrays) with potential to distinguish dietary groups in both male and female rats. Classification and predictive power were addressed using a series of megavariate data analysis approaches in both open and blinded analyses across the lifespan and across different extents of nutritive intake. Notably, we found that the use of appropriate algorithms allowed us to distinguish such diets across several years, even when the signal due to caloric intake was apparently swamped by the cohort-cohort differences observed. We have now begun to adapt these marker profiles for use in human epidemiology studies. In particular, we have shown that we can identify the majority of our markers in humans and that these can pass classical tests, such as blinded precision (analytical) tests, stability requirements, and tests examining inter- vs intra-individual variability. We will present the models, their ability to distinguish sera based on caloric intake, and the initial results of tests moving these markers to epidemiological studies in human plasma.

Keywords: caloric restriction, risk, breast cancer

278 Estrogen and IGF-I Crosstalk in Breast Cancer: Potential Therapeutic Implications

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The long-term goal of our Program Project is to understand major pathways in breast tumorigenesis. In recent years, it has become clear that estrogen receptor (ER) and progesterone receptor (PR) activity is dramatically influenced by growth factor signaling pathways, and that this cross-talk is a major determinant of both breast cancer progression and response to therapy. The understanding of mechanism and clinical implications of this crosstalk is the current focus of our studies. In this regard, we and others have shown intricate cross-talk between ER and insulin-like growth factor (IGF) pathways in breast cancer, although the exact mechanisms of cross-talk, and the importance on breast cancer outcome and response to therapy are poorly understood.

To better understand crosstalk between ER and IGF in breast cancer we identified and examined global gene expression changes following stimulation of breast cancer cells (MCF-7) with estrogen, IGF-I or the combination. Estrogen and IGF-I caused temporal changes in gene expression that were strongly associated with cell proliferation, metabolism, and DNA repair. Genes with early and sustained regulation by IGF-I were highly enriched for transcriptional targets of the estrogen receptor (ER), ras/ERK1/2, and PI3K/Akt/mTOR pathways. We found significant overlap between genes regulated by estrogen and IGF-I, but found a large number of genes uniquely regulated by the combination of estrogen and IGF-I. Interestingly, mRNA levels of genes co-regulated by estrogen and IGF-I were actually decreased rather than increased. There has been a recent appreciation for estrogen-mediated repression of gene expression, and in studying this further we found that ER is recruited to a number of promoters and represses transcription via a novel interaction with the understudied histone deacetylase HDAC7.

To investigate the biological importance of these studies we examined the gene expression profiles in human breast cancer. Many studies have recently focused on estrogen-induced gene expression profiles, and so we initially examined the IGF-I signature. In three large independent datasets of profiled human breast tumors, the IGF-I signature was manifested in the majority of ER-negative (ER-) breast tumors, however it was also found in subset (~25%) of ER+ breast tumors that had low levels of ER, suggesting a possible involvement in the transition to hormone independence. Indeed, patients with ER+ tumors that manifested the IGF-I signature had poor prognosis. The IGF gene signature was highly correlated with numerous poor prognostic factors (tumor size, hormone receptor status, HER-2 status, nodal status, grade), but in multivariate analysis it was one of the strongest independent indicators of disease outcome. The concept of cross-talk between estrogen and IGF-I, and the notion that IGF-I may be important in the transition to hormone independence is now being tested in the ongoing Phase 2 trial in postmenopausal women with advanced ER-positive disease comparing the aromatase inhibitor exemestane versus exemestane and an anti-IGF-IR blocking antibody (CP-751,871, Pfizer). Integrated biomarker studies will investigate the role for IGF-IR signaling in hormone response in breast cancer.

Keywords: estrogen, IGF, breast cancer

279 Copy Number Gain at 8q22 in Breast Cancer Is Associated With Poor Outcome and Chemoresistance

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High-copy chromosomal amplifications, containing a limited number of genes, are less common in human breast cancer than low-copy gains, containing many genes. It is more difficult to assign a pathogenic role to these low-copy gains; most attempts have used a combination of genomic and functional approaches to connect copy number gain, over-expression of gene products, and their functional effects on cell and tumor behavior.

We studied 129 primary, sporadic human breast cancers from women treated in the Dana-Farber/Harvard Cancer Center and collected by the Harvard SPORE in Breast Cancer and subjected to gene expression profiling with Affymetrix U133 arrays. Thirty nine tumors were microdissected and DNA from the tumors and matched normal tissue hybridized to Affymetrix 10K SNP arrays. Clinical outcome through a minimum of 36 months was available for 115 cases; 77 were treated after surgery with an anthracycline-based regimen with or without taxanes. Breast cancer cell lines with or without the 8q22 gain were treated with a panel of chemotherapeutic drugs and with gene-specific or control siRNA and analyzed for growth, apoptosis and for the cellular distribution of doxorubicin as described in the text.

Integrated DNA and expression array analysis in primary human breast tumors identified chromosome 8q22 copy number gain and a suite of over-expressed genes in this region. The 8q22 gain was confirmed by fluorescence in situ hybridization (FISH) and was present in 21% of tumors at a copy numbers between 3.5 and 8.0 per tumor nucleus. Parallel analysis of gene expression identified 114 probes differentially expressed between cases with or without distant metastatic recurrence within 36 months of diagnosis. Of these, 17 probes (15%) mapped to a nine megabase region centered at 8q22. The 17 probes correspond to 12 unique genes in a region with 75 known genes in total. This was the only cytogenetic region in the genome with a statistically significant enrichment of probes associated with metastatic disease. The finding of a poor outcome associated with an 8q22 copy number gain was corroborated using published cohorts of patients from the University of California San Francisco and from the Netherlands.

Three unique oligonucleotides targeting each of the 12 over-expressed genes were administered to cells harboring 8q22 gain (BT549) and to cells without gain (SKBR3). Knockdown of 4 genes by at least two of the three siRNA reduced viable cell number in BT459 but not in SKBR3. These genes include a lysomsome associated gene (LAPTM4B), 14-3-3zeta, GRHL2, and MTDH (metadherin). Unleashed apoptosis was the most prominent consequence of knockdown. We found depleting the expression of 2 of the 4 genes (tested so-far) resulted in increased sensitivity to doxorubicin but not to cisplatin. Finally, the intracellular distribution of doxorubicin was fluorescently monitored before and after depletion of LAPTM4B. Doxorubicin was significantly retained in the cytoplasm and excluded from the nucleus in cells with 8q22 gain and over-expression of LAPTM4B, and was more quickly admitted to the nucleus after LAPTM4B knockdown.

We are using an integration of approaches and technologies to evaluate regions of low copy gain at chromosome location 8q22 in breast cancers and breast cancer cells. Genomic and functional studies point to several genes, and we have begun to examine the consequences of their overexpression and experimental manipulation.

Keywords: copy number gain, amplification, outcome

280 14-3-3ζ in the Early Stages of Breast Cancer Progression: Luminal Filling and Epithelial Mesenchymal Transition

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Recent progress in diagnostic tools allows many breast cancers to be detected at an early pre-invasive stage. Thus, a better understanding of the molecular basis of early breast cancer progression is essential. Previously, we discovered that 14-3-3ζ is overexpressed in >40% of advanced breast cancers and this overexpression predicts poor patient survival. Here, we examined at what stage of breast disease 14-3-3ζ overexpression occurs and found that increased expression of 14-3-3ζ begins at atypical ductal hyperplasia (ADH), an early stage of breast disease. To determine whether 14-3-3ζ overexpression is a decisive early event in breast cancer, we overexpressed 14-3-3ζ in MCF10A non-transformed mammary epithelial cells (MECs) and established stable lines (10A.ζ). In a three dimensional (3D) culture model, the 14-3-3ζ overexpressing 10A.ζ MECs had severely disrupted acini architecture resulting in luminal filling. Proper lumen formation is a result of anoikis, apoptosis due to detachment from the basement membrane. We found that 14-3-3ζ overexpression conferred resistance to anoikis. Additionally, 14-3-3ζ overexpression in MCF10A cells and in MECs from 14-3-3ζ transgenic mice reduced expression of p53, which is known to mediate anoikis. Mechanistically, 14-3-3ζ induced hyperactivation of the PI3K/Akt pathway which led to phosphorylation and translocation of the MDM2 E3 ligase resulting in increased p53 degradation. Ectopic expression of p53 restored luminal apoptosis in 14-3-3ζ overexpressing MCF10A acini in 3D cultures.

Additionally, the 14-3-3ζ overexpressing 10A.ζ MECs demonstrated typical morphological alterations of epithelial-mesenchymal transition accompanied by loss of epithelial markers and gain of mesenchymal markers. Mechanistically, 14-3-3ζ overexpression activated TGFβ/Smads pathway, increased Smad-interacting protein 1 (SIP-1) expression that induced epithelial-mesenchymal transition. Importantly, patients with Ductal Carcinoma In Situ (DCIS) lesions that express both high 14-3-3ζ and TGFβRI showed at least two EMT marker alterations (reduced epithelial marker E-cadherin, and expressed mesenchymal marker vimentin and/or N-cadherin) and these DCIS were diagnosed as high-grade DCIS (grade 3) with a higher risk of developing invasive recurrence.

These data suggest that 14-3-3ζ overexpression is a critical event in early stages of breast disease. Downregulation of p53 is one of the mechanisms by which 14-3-3ζ alters MEC acini structure and contributes to breast disease initiation. 14-3-3ζ-mediated activation of TGFβ/Smads pathway and subsequent induction of EMT contributes to increased risk of DCIS progression to breast cancer. Our study identified 14-3-3ζ overexpression as a novel biomarker of high risk ADH/DCIS patients for more aggressive treatment at early stages. Moreover, it provides new targets for designing novel intervention strategies to prevent ADH/DCIS progression to invasive ductal carcinoma.

Keywords: 14-3-3ζ, p53, epithelial-mesenchymal transition

281 Tumor Self-Seeding Links Tumor Growth With Metastasis

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A strong association between tumor size and metastatic relapse is frequently observed in patients with breast cancer, but the basis for this link remains unknown. In conventional models, cancer progression is segregated into separate processes of tumor growth and metastasis, with a high rate of cell proliferation over cell death contributing to tumor expansion, and an accumulation of favorable alterations leading to metastasis without contributing to tumor growth. However, recent findings promote the idea that certain genes that specifically mediate distant metastasis may also promote primary tumor growth. A lung metastasis gene signature (LMS) was identified, the expression of which promotes both mammary tumor growth and lung metastasis.[1] Some of the genes included in the LMS favor tumor growth by promoting neoangiogenesis. Additionally, these genes support cancer cell extravasation from lung capillaries and other prometastatic functions.[2] Although current models would not regard such prometastatic functions as mediators of tumor expansion, we proposed that cancer cells expressing these functions could contribute to tumor growth through a mechanism of tumor self-seeding.[3] According to this hypothesis, aggressive circulating breast cancer cells can re-seed a mammary tumor, and the seeding of a tumor with its own circulating cells would accelerate tumor growth. We have recently obtained experimental evidence that supports this hypothesis by demonstrating that circulating metastatic breast cancer cells readily seed mammary tumors in a mouse model through the agency of metastatic extravasation genes. From privileged locations in the receiving tumor, the seeding cells subsequently accelerate tumor expansion by paracrine stimulation of neovascularization and tumor infiltration with stromal cell types. The present identification of molecular mechanisms and biological consequences of tumor self-seeding suggest an explanation for the association of tumor growth and metastasis. Moreover, this new model has major implications regarding the understanding of clinical phenomena, such as local recurrence following excision of primary tumors to "clean margins," and the design of improved anticancer drugs and drug schedules.

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Keywords: metastasis, self-seeding, Gompertzian

282 NSABP Biospecimen Bank

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The National Surgical Adjuvant Breast and Bowel Project (NSABP)

The NSABP has randomized more than 100,000 patients diagnosed with breast or colorectal cancer into clinical trials over the past 50 years. Based on incremental benefit achieved through multiple steps of evolution of treatment modalities tested in those trials, significant improvement in patient outcome has been achieved. However this also means that there is a significant over-treatment of patients who did not benefit from the various interventions. Development of reliable prognostic and predictive tests is required to deliver the promise of personalized treatment. Archived tumor tissue blocks from historical trials provide an essential resource to develop such tests since trials cannot be conducted again once efficacy of newer regimens is demonstrated due to ethical reasons.

The NSABP Biospecimen Bank serves as an open resource to the scientific community to provide tissues for development and clinical validation of new prognostic or predictive tests. Tissue specimens are provided to investigators from both academia and the private sector based on scientific merit.

Studies for prognostic or predictive tests are conducted with statistical rigor which requires the validation of the candidate algorithm in a completely independent clinical cohort that was not used for the development of the algorithm.

Gene expression profiling using QRT-PCR in collaboration with Genomic Health, Inc., has led to the development of OncotypeDx assay based on expression levels of 21 genes. This assay provides continuous estimates of risk of distant failure for patients diagnosed with estrogen receptor positive node negative breast cancer who are treated with tamoxifen and provides guidance as to the need for chemotherapy in addition to tamoxifen. A similar assay for colon cancer is undergoing validation.

In-house research efforts have resulted in development of methods for microarray gene expression profiling of formalin fixed paraffin embedded tumor tissue. The method is currently being utilized to discover predictors of the degree of benefit from trastuzumab added to adjuvant chemotherapy.

Keywords: tissue bank, gene expression, correlative science

283 Stromal Interaction and Migratory Pathways Identified in Invasive Tumor Cells Isolated From Human Breast Tumors

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Metastasis is a multicomponent process, with potentially different tumor cell properties and molecules of the motility pathways playing critical roles in the individual steps of metastasis (Nat Rev Cancer. 2007. 7(6):429-40, Nat Rev Cancer. 2003. 3(12):921-30). Therefore, the development of new molecular and imaging methods to identify new genes that contribute to specific cell behavioral steps in metastasis is crucial. We have used 2-photon intravital imaging to observe tumor cell invasion and intravasation directly in living mammary tumors and we have combined these observations with expression profiling to derive an invasion signature (ARCDB 2005 21:695). This was done in mammary tumor in both rats and mice (Cancer Res. 2007. 67(8):3505-11, Cancer Res. 2004. 64(23):8585-94.). But the human invasion signature has not been studied yet.

We are using the methods we developed in the rat and mouse models to investigate microenvironments in human breast tumor metastasis. We have previously shown in the mouse and rat mammary tumors that infiltrating macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor (CSF-1/EGF) paracrine loop (Cancer Res. 2007. 67(6):2649-56, Cancer Res. 2004 64:7022). In this study we show *in vivo* that invasion in a human breast cancer xenograft model, unlike its mouse and rat counterparts, is driven by both an autocrine regulation of the CSF-1 receptor in the cancer cells themselves, as well as a paracrine interaction with the macrophages inside the primary tumor through the CSF-1 / EGF loop. Moreover, the invasive human tumor cells were compared to the average tumor cells of the same primary tumor for their expression patterns of genes predicted to play important roles in invasion and metastasis, based on our previous studies in mouse and rat models. Through quantitative PCR, we show changes in the expression of genes that occur uniquely in the invasive subpopulation of human tumor cells. Specifically, components of the Arp2/3 complex, the actin capping proteins, and components of the cofilin pathway are differentially regulated in the invasive tumor cells compared to the resident tumor cells of the primary breast tumor. In particular, the expression of the anti-capping protein Mena is greatly up regulated leading to enhanced sensitivity to EGF. Mena over expression is correlated with increased metastasis of breast cancer patients as determined in a retrospective study of 60 patients with known outcomes. Our results indicate that the patterns of expression of motility-related genes that contribute to invasion and metastasis exist in the human breast tumors, similar to their mouse and rat counterparts. Additional verification of these results in tumors from patient-derived samples, as well as a whole genome microarray comparison of the invasive versus resident primary tumor cells, is underway. Ultimately, the discovery of gene expression patterns involved in invasion and the formation of autocrine and paracrine loops in human tumors will provide new therapeutic targets and diagnostic markers.

Keywords: metastasis prognostic marker, human invasion signature, autocrine and paracrine loops of invasion

284 Mining Cancer-Specific Alterations in Alternative Splicing and Glycosylation for Potential Biomarkers That Enable the Early Detection of Metastasis-Prone Breast Cancers

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Introduction: We are members of the Clinical Proteomic Technologies Assessment for Cancer (CPTAC) consortium for benchmarking unbiased and targeted mass spectrometry (MS)-based approaches to enable the early detection of cancer. We are also mining cancer-associated changes in co- and post-translational modifications as a source of candidate biomarkers for further evaluation using said platforms. There are several reasons that we chose this approach. First, we discovered, using exon specific microarray analysis, that specific changes in mRNA splicing are associated with breast cancer subtypes resulting in the production of distinct subtype-specific protein isoforms. Additionally, we knew that many biomarkers used clinically are carbohydrate structures that are carried by many different proteins, rather than single species, which amplifies the signal.

Alternative Splicing: The interrogation of splicing on the whole-genome scale is feasible using exon level expression data measured using microarrays. We performed a global analysis of splice variants in breast cancer by profiling over 30 breast cancer cell lines using two independent microarray platforms: the Affymetrix Human Exon 1.0 ST arrays and a prototype of the new Affymetrix Research Human Junction Array. We also developed a novel computational method for detection of splice variants in these data sets. This analysis identified ~1700 genes that showed strong evidence of alternative splicing across all cell lines. Next, we applied standard t-tests on the Splicing Index scores to detect splice variants highly expressed either in the basal (metastasis-prone) or the luminal subclasses. Using a p-value cut-off of 0.01 we selected the top 31 internal exons within 30 genes that showed the largest differential rate of exclusion/inclusion between basal and luminal cells. Validation is now underway and subtype specificity has been confirmed for CD44, FLNB, CLTC, PLEC1, FAT, FER1L3, DST, SLK, MYO6, and FAM62B. Protein isoforms specific to invasion prone basal subtype cancers are ideal early detection candidates for analysis by using MS approaches since they yield distinctive proteins that should be preferentially expressed in metastasis-prone breast cancers. **Glycosylation:** We analyzed 44 breast cancer cell lines for the expression of specific cell surface glycoconjugates that could be markers of the metastasis-prone subtype. Novel classes were localized by immunostaining of cells grown on microscope cover slips using monoclonal antibodies to specific carbohydrate epitopes. In addition, glycoproteins that carried these epitopes were visualized by immunoblotting of corresponding cell lysates. Several interesting results emerged. For example, many basal-type cancer cells expressed the sulfated high-affinity L-selectin carbohydrate ligand recognized by the MECA-79 monoclonal antibody. This unusual oligosaccharide is an important component of the mechanism that mediates leukocyte rolling and tethering, the first step in extravasation from blood. We also detected expression of the MECA-79 epitope by cancer cells in breast biopsies from 20 cases; surrounding normal tissue did not stain. As to the identity of the glycoproteins carrying the MECA-79 epitope, an immunoprecipitation (IP; anti-CD44) and immunoblot strategy (IB; MECA-79) showed that a subset of the CD44 isoforms carried this carbohydrate epitope and the results were confirmed by reversing the reagents that were used for IP and IB. Other experiments suggest that MUC-1 is another MECA-79-reactive glycoprotein. Regarding the possible functions of these unusual sulfated/fucosylated structures, additional data suggest that their interactions with selectins could play a crucial role in the haematogenous spread of cancer cells.

Conclusions: Our pipeline enables the identification of candidate biomarkers of metastasis-prone breast cancers, whose clinical utility can be further evaluated by applying state-of-the art MS approaches to the analysis of patient and control plasma samples.

Keywords: breast cancer, alternative splicing, glycosylation

285 Identification of Predictors of Response in a Phase II Trial of Preoperative Cis-platinum in Early Stage Triple-Negative Breast Cancer

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Laboratory data suggested that tumors deficient in BRCA1 may be more susceptible to the DNA damaging agent cis-platinum. Genomic characterization demonstrated marked similarities between BRCA1-associated and sporadic triple-negative breast tumors (negative for estrogen receptor (ER), progesterone receptor (PR) and HER2). In a related study, ΔNp63 was found to control a pathway for TAp73-dependent cisplatin sensitivity that was specific to triple-negative breast tumors. We conducted a phase II trial of preoperative cis-platinum in 28 patients with early stage triple-negative breast cancer. Pathologic response was measured using the Miller-Payne (M-P) system based on radiologic-pathologic assessment of tumor reduction after chemotherapy. Six of 28 (22%) achieved pathologic complete response (M-P score 5), including both carriers of germline BRCA1 mutations. Overall, 14 (50%) were sensitive (M-P scores 3,4,5), and 14 (50%) were resistant (M-P 0,1,2) to cisplatin. Frozen tumor tissue obtained prior to initiation of chemotherapy was available for 24 of the 28 patients. Assays were performed for germline BRCA1 mutation, tumor BRCA1 expression and promoter methylation, p53 mutation, and ΔNp63/TAp73 expression ratio. Pretreatment tumor samples were also analyzed by gene expression array profiling and MIP array DNA profiling.

Hierarchical clustering of gene expression profiles show that 23 of 24 tumors cluster with other basal-like tumors in a reference cohort. One case clustered with HER2 positive tumors and was resistant to cisplatin; this case was HER2 2+ by IHC and FISH not amplified. Supervised learning identified no single gene with significant association with cisplatin response after Bonferroni correction for multiple comparisons. Published gene signatures for E2F3 overexpression, higher chromosomal instability, and proliferation were weakly associated with cisplatin sensitivity.

Sequencing confirmed known germline BRCA1 mutations,in two patients, and no additional germline mutations were identified. RT-PCR measurement showed a trend for lower BRCA1 expression levels in sensitive tumors and combination of low BRCA1 and high Ki67 expression identifies 7/11 sensitive and 1/10 resistant tumors (chisq 6.39, $p = 0.012$). BRCA1 expression is correlated with expression of neighbor of BRCA1 gene 2 (NBR2) which shares the same promoter region, suggesting alteration of the promoter may underly the reduced expression levels.

P53 sequence demonstrated 6 wild type, 11 substitution mutations, and 5 truncating mutations. Truncating p53 mutations were significantly associated with sensitivity to cisplatin. A ratio of ΔNp63/ TAp73 > 2 (biomarker positive) was found in 6/11 sensitive and 2/10 resistant tumors (chisq 2.65, $p=0.1$) and the p63/p73 biomarker was positive in 3/4 tumors with pathologic complete response, supporting a possible trend for association with cisplatin sensitivity.

In summary, we have clinical evidence to suggest low BRCA1/high Ki67, ΔNp63/ TAp73 > 2, and p53 truncating mutations are biomarkers associated with cisplatin sensitivity. These will be tested in a second cisplatin-based preoperative treatment trial that is currently underway.

Keywords: triple-negative, cis-platinum, response biomarkers

286 Influence of the Wound Microenvironment on Tumor Growth

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Chronic (skin) wounds and inflammation are well known risk factors for cancer. In a Rous sarcoma virus tumor model it has been shown that wounding induces tumor growth at the wounded site. Although tumors ultimately destroy the integrity of tissues whereas wound healing repairs tissues, wounds and tumors have striking similarities, and tumors are described as “wounds that do not heal”.

We investigated the influence of a local or remote wound microenvironment on tumor growth and the contribution of the immune system to wound promoted tumor growth in a syngeneic mouse breast cancer model. Metastatic mouse breast cancer cells (4T1) were orthotopically injected into the mammary fat pads IV/V of BALB/c mice. Animals were wounded locally by 10mm full thickness dermal incisions above the mammary fat pads IV/V or remotely by 1mm dermal incisions above the scapula 9 days after tumor cell inocculation, shortly before tumors became palpable. Tumor volumes were monitored by measuring the 2 main axes of the tumor with calipers. Animals were euthanized 23 days after tumor cell inoculation and tumor tissue and inner organs were procured for histological analysis.

Local wounding as compared to sham treatment increased tumor size over the entire course of the experiments, whereas remote wounding at the shoulder had little or no effect. Similarly, injection of wound fluid into the tumor site increased tumor growth compared to serum or DPBS. In BALB/c nu/nu mice that lack the T-cell compartment local wounding had little or no effect on tumor growth. Consistent with our in-vivo data in-vitro results showed that wound fluid as compared to serum increases the proliferation rate of 4T1 cells by 29% ($p=0.049$). Wound fluid as compared to serum also increased migration and invasion of 4T1 cells, and, similarly, wound fibroblasts as compared to dermal fibroblasts increased migration of tumor cells in Boyden chamber assays.

Our results show that wound stroma can influence development of nearby tumors in an unfavorable way. This effect is T-cell dependent and can be mediated by wound fluid, indicating that T-cell secreted cytokines or growth factors are involved in wound promoted tumor growth.

Keywords: breast cancer, wound stroma, immune system

287 Analysis of B-Myb in Basal-Like Breast Cancer

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Breast cancer consists of several distinct subtypes based upon gene expression profiling, clinical behavior and the repertoire of expressed therapeutic targets. The breast cancer subtypes include luminal A and B, normal-like, HER2-enriched, claudin-low and basal-like, each having unique gene expression profiles and distinct clinical outcomes. A defining feature of basal-like breast cancer, a subtype with poor clinical prognosis, is the high expression of "proliferation signature" genes. We identified *B-Myb*, a MYB family transcription factor that is often amplified and overexpressed in a variety of tumor types, as being highly expressed in the proliferation signature. However, the roles of B-Myb in disease progression, and its mammary-specific transcriptional targets, are poorly understood.

Here, we demonstrated that *B-Myb* expression is a significant predictor of pathological complete response to neoadjuvant chemotherapy and poor survival. We also identified a significant association between the G/G genotype of a nonsynonymous *B-Myb* germline variant (rs2070235, S427G) and an increased risk of basal-like breast cancer [OR 2.0, 95% CI (1.1-3.8)]. Interestingly, the *B-Myb* variant was found to be nearly ten-fold more frequent in African-Americans (7%) versus non-African-Americans (0.8%) in the CBCS. This is relevant in light of recent data showing that premenopausal African-Americans are approximately twice as likely to develop basal-like tumors compared with premenopausal Caucasians.

We also found that immortalized, human mammary epithelial cell lines overexpressing *B-Myb* or S427G variant showed increased sensitivity to two DNA topoisomerase IIα inhibitors, but not to other chemotherapeutics. In addition, microarray analyses identified many G2/M genes as being induced in *B-Myb* overexpressing cells. These results confirm that B-Myb is involved in cell cycle regulation, and that dysregulation of *B-Myb* may contribute to increased sensitivity to a specific class of chemotherapeutic agents. These data point to B-Myb as a biomarker in human breast cancer that is of potential clinical importance for evaluating disease risk and for guiding treatment.

Keywords: breast cancer, B-Myb, basal-like

288 Modulation of Breast Cancer Progression by BNIP3

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Background: Tumor hypoxia and necrosis are tightly correlated with metastasis and poor prognosis in breast cancer, although the molecular basis of this correlation is not well defined. BNIP3 is a hypoxia-inducible regulator of autophagy and cell death whose expression is up-regulated in various human cancers as they outgrow their supply of oxygen and nutrients.

Materials & Methods: We examined BNIP3 expression during the progression of primary human breast cancer by immunohistochemical staining of tumor microarrays representing different stages of disease progression. We have pursued the mechanistic basis of observed changes in BNIP3 expression through structure-function analyses in tumor cell lines and in mouse models to ascertain how altered BNIP3 expression affects breast cancer progression.

Results: We observed altered sub-cellular localization of BNIP3 during human breast cancer progression such that BNIP3 switched from a predominantly cytoplasmic localization at non-invasive stages of disease to nuclear as tumors progressed to become invasive. We have identified a nuclear export signal within the trans-membrane domain of BNIP3 that mutagenesis studies implicate in promoting the export of BNIP3 from the nucleus to the mitochondria where it functions to promote mitochondrial turnover in response to oxidative stress. Conversely, the import of BNIP3 to the nucleus appears to be modulated by phosphorylation on key residues. On-going studies are examining the consequences of nuclear BNIP3 expression for tumor cell growth and viability, and for acquisition of invasive properties following injection of engineered cells into mouse mammary fat pad and through the use of genetically engineered mouse models.

Discussion: Our work suggests that the sub-cellular localization of BNIP3 may be a useful molecular marker of breast cancer progression. Furthermore, our work indicates that inactivation of BNIP3 through nuclear sequestration may play a role in promoting tumor progression in response to hypoxia, by preventing normal turnover of mitochondria by autophagy that is required to prevent accumulation of reactive oxygen species and necrosis.

Keywords: hypoxia, necrosis, tumor progression

289 Development of a Membrane Microfilter Device for Capture and Characterization of Circulating Tumor Cells (CTC) in Blood

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Isolation of CTC from human blood currently poses significant challenges to the evaluation of tumor metastasis. Although there have been a number of technologies designed to improve the ability to identify and monitor CTC in the blood of cancer patients, difficulties with sensitivity, specificity, efficiency, and high costs of materials and reagents continue to limit these efforts. Here, we present a novel membrane microfilter device for isolation of CTC in blood by exploiting size differences between tumor and normal blood cells. We evaluated the sensitivity and efficiency of CTC capture in a model system, and compared the membrane microfilter device with the CellSearch platform, recognized as the "gold standard" for isolation of CTC, in blood samples from patients with cancer.

For the model system, 5 cultured human cancer cells were directly micro-pipetted into 7.5 ml of whole blood from healthy, cancer-free donors, and processed by the membrane microfilter device. 58 trials of this experiment were performed; 29 in which the J82 bladder cancer cell line (known to have a characteristically small diameter relative to other epithelial cancer cell lines) were used, and 29 in which tumor cells from a mixture of 6 different human cancer cell lines (J82 and T24 bladder, MCF-7, SK-BR-3 and MDA-MB-231 breast, and LNCaP prostate) were used to simulate maximal size heterogeneity. In addition, 7.5 ml blood samples were analyzed from 49 patients with prostate cancer: 21 with confirmed metastasis from Memorial Sloan-Kettering Cancer Center (MSK), New York, NY, and 28 with status of metastasis undisclosed from the University of Chicago Medical Center (UC), Chicago, IL, using the membrane microfilter device. 7.5ml of blood from all patients at MSK were also processed by the CellSearch platform. The membrane microfilter device successfully recovered $\geq$1 tumor cell in 96.5% (28/29) and 93.1% (27/29) trials where 5 J82 cells or 6 tumor cell types were used, respectively, and recovered 3 or more cells in 64% of these trials. Statistical analyses confirms that the true chance of recovering at least 1 tumor cell when 5 are seeded from 7.5ml of blood is 95% or greater. In clinical samples, the membrane microfilter device successfully recovered CTC from blood in 100% (21/21, 14-182 cells recovered) of patients with metastatic prostate cancer from MSK. In contrast, the CellSearch platform recovered CTC in 57% (12/21, 0-140 cells recovered) in corresponding blood samples. When CTC were detected by both methods, greater numbers were recovered by the membrane microfilter device in all but 3 patients. Of the 28 samples analyzed from UC, the microfilter device detected CTC in 74% (17/23) patients with confirmed metastasis. CTC were detected in 20% (1/5) prostate cancer patients with no evidence of metastasis. Multimarker immunofluorescence (IF) can be performed and evaluated directly on the membrane microfilter device, with up to 4 IF markers simultaneously assessed utilizing quantum dots as labels. We have successfully developed a quadruplexed assay for CD44, CD24, ALDH-1, and Pan Cytokeratin in breast cancer cell lines captured by the device.

Our data demonstrates that the sensitivity and efficiency of CTC isolation by the membrane microfilter device compares favorably to the current "gold standard" CellSearch platform. The membrane microfilter device has transformative potential to provide a cheaper, faster, and better alternative to current approaches to CTC isolation, and does not depend on affinity based capture of cells, as do other platforms. A major advantage is that cell characterization can take place directly on the device. Future efforts using the membrane microfilter device will focus on the enumeration of CTC as an early indicator of therapeutic efficacy, and on the biological characterization of CTC, including identification of therapeutic targets, using multimarker IHC/IF, FISH, PCR and other techniques directly on the device. Further uses of the membrane microfilter device include improved diagnosis of bladder cancer by microfiltration of urine, an idea we are currently testing.

Keywords: circulating tumor cells, tumor metastasis, prostate cancer

290 The Low Molecular Weight Forms of Cyclin E are Tumor Specific in Breast Cancer and Tumorigenic in Human Mammary Epithelial Cells

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Background: Low molecular weight (LMW) isoforms of cyclin E are post-translationally generated in breast cancer cells and are linked to poor prognosis. Despite their correlation with aggressive disease, the direct role of the LMW isoforms of cyclin E in mediating tumorigenesis remains unclear.

Methods: Cyclin E was measured in tumor and non-tumor tissue from breast cancer patients to understand the relationship between full-length and LMW cyclin E. Biacore analysis was performed to assess biochemical differences between the cyclin E isoforms and CDK2. Non-tumorigenic (76NE6) mammary epithelial cells transfected with the cyclin E isoforms were used to study the biologic consequences of the LMW isoforms of cyclin E in vitro and in mice.

Results: The LMW isoforms of cyclin E are tumor specific as they are detected much more significantly in tumor tissue from breast cancer patients than in normal adjacent tissues ($p<0.0001$). They have increased efficiency of binding to CDK2 and result in increased clonogenicity and inability to enter quiescence in response to growth factor deprivation, compared to the full-length cyclin E. Additionally, 76NE6 cells overexpressing LMW cyclin E are genomically unstable. Furthermore, in a xenograft model, we were able to show that the LMW isoforms of cyclin E are directly involved in the tumorigenic process as the LMW cyclin E gave rise to tumors, whereas full-length cyclin E did not. Lastly, the expression of LMW cyclin E is is found to be most prevalent in triple negative breast cancers (i.e. $p=2.83\ 10^{-6}$).

Conclusion: The LMW isoforms of cyclin E are distinct from the full-length cyclin E, both biochemically and biologically, and are directly involved in the tumorigenic process of breast cancer.

Keywords: cyclin E, low molecular form, tumorigenic

291 Activation of Caspase-Independent Programmed Cell Death Overcomes Apoptosis Resistance in Ovarian Cancer Cells

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Introduction: Chemoresistance is a major burden in the treatment of patients with ovarian cancer and is associated with failure to induce apoptosis. In pan–resistant cancer cells, the full induction of the apoptotic cascade is inhibited due to high levels of expression of anti-apoptotic proteins, which prevent caspase activation. The objective of this study was to evaluate whether the activation of a caspase-independent alternative pathway could promote cell death in chemo-resistant ovarian cancer cells. We describe the characterization of a novel compound, NV128, which promotes cell death in a caspase-independent manner in chemo-resistant ovarian cancer cells.

Methods: Eight primary cultures and two established epithelial ovarian cancer (EOC) cell lines were treated with increasing concentrations of NV128 (0.1, 1, and 10 μg/ml) with or without the pan-caspase inhibitor, Z-VAD-FMK. Cell viability was determined after 24h using the Celltiter 96 assay. DNA fragmentation was analyzed by flow cytometry with Hoechst and Propidium iodide staining. Activity of caspases- 3/7, -8, and -9 was measured using Caspase-Glo assay. Protein expression was determined by Western blot analysis.

Results: NV128 treatment decreased cell viability in all tested EOC cells lines in a dose-dependent manner with IC_{50} between 1 and 5 μg/ml. Flow cytometry analysis revealed DNA fragmentation, with >90% cells staining double-positive for Hoechst and Propidium iodide after 24h. Cell death was however, caspase-independent as evidenced by the lack of caspases- 3/7, -8, and -9 activity and the inability of the pan-caspase inhibitor, Z-VAD-FMK, to prevent cell death. DNA fragmentation was observed as the result of the activation of an intracellular pathway involving: down-regulation of pAKT, cleavage of LC3 to LC3-II, Beclin mitochondrial translocation leading to Bcl2 inhibition, and nuclear translocation of EndoG.

Conclusion: We describe an alternative pathway leading to DNA fragmentation and cell death, which does not depend on caspase activation. Our findings demonstrate the possibility of using therapeutic drugs, such as NV128, which could overcome resistance to the classical caspase-dependent apoptosis and therefore have beneficial effects in chemo-resistant ovarian cancer patients.

Keywords: ovarian cancer, mTOR, caspase independent

292 Optimizing a Two-Stage Strategy for Early Detection of Ovarian Cancer

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Five year survival for ovarian cancer patients has increased significantly over the last three decades, but rates of long-term survival have not changed, related, in large part, to diagnosis at a late stage in >70% of patients. Up to 90% of patients can be cured when disease is detected in stage I, whereas less than 30% of patients are cured in stage III-IV. Our group is optimizing a two-stage strategy for early detection of ovarian cancer, funded by the U.T. M.D. Anderson Ovarian Cancer SPORE with help from several foundations. Given the prevalence of ovarian cancer in the post-menopausal population (1 in 2,500) any screening strategy must have high sensitivity for early stage disease (> 75%) and very high specificity (>99.6%) in order to achieve a positive predictive value of 10% (10 operations for each case of ovarian cancer detected). While a single determination of CA125 lacks the requisite sensitivity and specificity, greater positive predictive value can be attained by performing transvaginal sonography (TVS) in a fraction of apparently healthy women with rising CA125. In a translational SPORE screening study, postmenopausal women at average risk for ovarian cancer have undergone annual evaluation with serum CA125. Changes from year to year are analyzed with a computer algorithm. If CA125 rises significantly, patients are referred for transvaginal sonography (TVS) and, if indicated, for surgery. Over the last 7 years, 2,319 apparently healthy women have been screened at 6 different sites with 7,201 CA125 determinations. Less than 2% of the women have been referred for TVS and 5 patients have been referred for operations that have detected three ovarian cancers: a stage IA borderline and stage IIA and IIC invasive cancers. From these data, we predict that this screening strategy is likely to have a positive predictive value of greater than 14%. Among the women screened to date, we failed to detect one stage I borderline cancer, but have not missed an invasive ovarian cancer. Our results to date are consistent with those obtained in the UKCTOCS trial that involves 200,000 women in the United Kingdom and is adequately powered to demonstrate an improvement in survival.

As only 80% of ovarian cancers express significant levels of CA125, multiple markers may be required to detect all early stage disease. Multiplex assays have been used to measure 88 candidate biomarkers at the Pittsburgh Cancer Center. The multi-marker panel that provided the highest diagnostic power for both early and late stage disease was comprised of four biomarkers: CA125, HE4, s-EGFR and sVCAM-1. An algorithm that contains these markers achieved a sensitivity of 90% at 98% specificity in an independently collected validation set consisting of sera from 64 patients with stage IA ovarian cancer and 150 healthy women. Similarly, a proteomic panel has been identified using SELDI-TOF-MS at M.D. Anderson and Vermillion that includes transthyretin, Apo-A1 and CTAP3. In an independent GOG validation set of sera from 136 patients with stage I ovarian cancer, an algorithm that included the three proteomic markers in combination with CA125 produced a sensitivity of 87% at 98% specificity. Each of these panels is currently being tested with pre-clinical samples from the PLCO trial. Based on these results, an optimal panel will be chosen. Year-to-year variation in marker values can then be assessed with 5 annual samples from 200 healthy women, preserved from the SPORE screening trial. Sensitivity of the biomarker panel for early stage ovarian cancer can also be measured with sera from patients with Stage I disease, permitting the development of a new algorithm that measures the course of multiple markers over time. The specificity and positive predictive value of this novel algorithm can then be assessed in the next SPORE screening trial, using the consortium developed to evaluate CA125 alone.

Keywords: ovarian cancer, CA125, biomarker

293 Detection of MUC4 mRNA in Human Peripheral Blood Mononuclear Cells: Prognostic and Diagnostic Marker for Patients With Pancreatic Cancer

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Background: Pancreatic cancer (PC) is a highly lethal disease with a dismal 5-year survival rate of less than 5% chiefly owing to an inability to diagnose it at a localized and hence resectable stage. There is an urgent need for biomarkers that can aid in its early detection and to stratify patients for curative or palliative therapy. MUC4 is a high molecular weight mucin that is not expressed by the normal pancreas but aberrantly expressed at very high levels in pancreatic cancer tissues and well-differentiated pancreatic cancer cell lines. Preliminary results indicated that the detection of *MUC4* mRNA in peripheral blood mononuclear cells (PBMCs) could be a novel marker for the diagnosis and prognostication of pancreatic cancer patients. Thus, we further investigated the utility of this marker in combination with the CA19.9 marker, which is currently used to monitor tumor progression.

Methods: We quantified the levels of *MUC4* mRNA in PBMCs of 273 cases including 152 cases of pancreatic adenocarcinoma and 33 healthy controls by one step real time quantitative PCR. We also measured the levels of CA19.9, the most commonly employed marker in pancreatic cancer diagnosis and follow-up, by radioimmunoassay in 219 of these patients.

Results: *MUC4* mRNA levels were significantly higher in pancreatic cancer cases compared to healthy controls ($p<0.0001$) and those with pancreatitis ($p<0.0001$). When a cut-off of >0 for the normalized MUC4 transcript ratio (MUC4 index) was selected (diagnostic sensitivity= 70%), the diagnostic specificity of MUC4 for pancreatic cancer was 83.13 %. CA19.9 at a cut-off of 56.24 U/ml had the optimal sensitivity and specificity of 61.2% and 97.6% respectively, while at 37 U/ml, it was more sensitive (68%) but less specific (83%). A combined criteria of either a MUC4 index >0 or serum CA19.9 >37 gave a sensitivity of 92 % (specificity of 68.7%) to distinguish pancreatic cancer from healthy individuals and benign pancreatic diseases. Pre-operative high *MUC4* levels in PBMCs were significantly correlated with a shorter survival (r= -0.789). On following *MUC4* levels before and after surgery in a group of patients, there was a trend towards decline following removal of the tumor although this did not reach statistical significance. In univariate Cox survival analysis, a MUC4 index >0, advanced stage (AJCC stages III and IV) and CA19.9 >200U/ml were associated with an increased risk of death (hazard ratio =3.71, 3.07 and 3.71 respectively, $p<0.05$) in patients diagnosed with pancreatic cancer. This prognostic value remained significant for overall survival in the multivariate analysis. Kaplan-Meier survival curves demonstrated similar findings for *MUC4* mRNA levels. A CA19.9 value >200 U/ml, but not one > 37 U/ml was associated with reduced overall survival ($p=0.015$ and 0.35 respectively). *MUC4* mRNA levels in PBMCs (but not serum CA19.9) showed a modest correlation (correlation coefficient= 0.56, p value <0.05) with MUC4 expression in pancreatic cancer tissues for a set of nineteen pancreatic cancer patients.

Conclusion: The detection *MUC4* mRNA levels in PBMCs (MUC4 index >0) has a strong and independent unfavorable prognosis for patients with pancreatic adenocarcinoma. Further, in combination with serum CA19.9 levels, quantitative measurement of *MUC4* mRNA in PBMCs has the potential to be useful in the diagnosis of this belligerent malignancy. CA19.9 level >56.2 U/ml has better diagnostic accuracy than the more common cut-off of 37 U/ml, while levels >200 U/ml are strongly associated with poor prognosis.

Keywords: pancreatic cancer, diagnosis, MUC4

294 Using Epigenetic Biomarkers as Prognostic and Predictive Markers in Non-Small Cell Lung Cancer (NSCLC)

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Epigenetic gene silencing mediated through aberrant DNA methylation and histone deacetylation is a key contributor to lung carcinogenesis. We have defined the utility of a panel of promoter region DNA methylation markers as a robust potential molecular system for re-staging stage 1 NSCLC to stage 3 disease. We performed a blinded, retrospective, nested case control study of 167 patients who underwent curative surgery for stage 1 lung cancer (51 cases who recurred within 40 months; 116 controls who did not recur). The finding that 2 or more DNA hypermethylated genes in tumor plus histologically tumor-free mediastinal nodes can predict recurrent disease with odds ratios up to15-fold, constitutes a new paradigm for the molecular staging of lung cancer. The significance of these discoveries has important implications since silencing of these gene markers represent not only prognostic markers, but also these genes may serve as potential targets for a unique new adjuvant approach for stage 1 NSCLC using epigenetic therapy to re-express the silenced genes. To determine if targeting epigenetic changes in lung cancer patients improves the disease-free and overall survival of patients with metastatic lung cancer, we enrolled ten patients in a phase I trial combining inhibitors of DNA methyltransferase (5-Azacytidine (5AC)) with inhibitors of histone deacetylase (SNDX-275). This trial was limited to subjects ≥ 18 years of age with histologically confirmed recurrent NSCLC with progressive disease after at least one previous chemotherapy regimen. 5AC was administered SQ days 1-6 and 8-10 with SNDX-275 given days 3 and 10 of a 28 day schedule. SNDX-275 was administered at a fixed oral dose of 7 mg. 5AC dose was varied between cohorts. A standard 3+3 phase I dose assessment schema was used. One patient has had a durable, substantial response by RECIST criteria that has lasted over 14 months. This single patient had recurrent disease after surgery with curative intent for stage 1 disease and had ≥2 DNA hypermethylated genes from our previously defined gene panel in both her primary tumor and histologically tumor-free mediastinal lymph nodes. A second patient has had disease stabilization that has persisted over 8 months. The remaining patients had progressive disease.

Keywords: epigenetics, therapy, lung cancer

295 Pivotal Role of Translational Research in the Development of Novel Cancer Treatments by Conduct of Phase I Clinical Trials

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Modern, mechanism-based therapeutic agents are designed to act on molecular targets that are causally involved in the malignant progression of human cancers. They include immortality, growth signal autonomy, insensitivity to antigrowth signals, resistance to apoptosis, sustained angiogenesis, and metastatic potential. An integral aspect of the development of these new agents compared to the empirically developed cytotoxics is the implementation of new early clinical trial designs to include translational research with "proof of principle mechanistic analyses". Additionally, the measurement of pharmacodynamic (PD) endpoints allows for optimization of therapy in a patient-tailored approach. The Karmanos Cancer Institute (KCI) has therefore established a Biospecimen Facility and a Translational Research Laboratory to support targeted drug development in early phase clinical trials. These resources allow the development and implementation of hypothesis-driven clinical trials. Over the past three years, five NCI-CETP sponsored phase I trials with PD components were developed at KCI including small molecules and inhibitory antibodies that aim to overcome resistance to apoptosis and growth signal autonomy. Two of these agents, namely Aminoflavone Prodrug 464 (AFP464) and the Poly(ADP-Ribose) polymerase (PARP) inhibitor ABT-888, will be highlighted in our presentation. Both drugs can selectively induce DNA-damage signaling in certain “sensitive” tumor cells compared to normal cell types, but not all tumors will respond, hence necessitating the identification of markers that would allow us to preselect patients for treatment and to monitor response correlates.

In order to identify patients that might best benefit from AFP464 or ABT-888 treatment we used panels of human tumor cell lines, defined the drugs’ target levels, and correlated them to antiproliferative activity in these cells *in vitro*. In the case of AFP464 we found that cell lines expressing estrogen receptor and/or exhibiting cytosolic aryl hydrocarbon receptor (AhR) expression are exquisitely sensitive to AFP464, whereas cells with nuclear AhR are resistant. AFP464 selectively induces DNA-damage in sensitive tumor cells. For the PARP inhibitor ABT-888 we observed that it has little activity as a single agent in most tumor cells; however tumors with severe defects in DNA repair pathways (e.g. mutant p53, mutant BRCA1 or 2) exhibit single agent activity and are also very susceptive to combination of the PARP inhibitor with the topoisomerase I inhibitor irinotecan.

The information resulting from these translational studies has been used by us to define parameters that will be analyzed as possible markers of response in the clinical protocols. In addition, we have established the detection of phosphorylated histone 2AX (γ-H2AX) as a PD endpoint marker of early DNA-damage signaling for AFP464 and the combination of ABT-888 with irinotecan. The advantage of using γ-H2AX is that it can be easily detected by immunofluorescence as typical intense nuclear foci in few tumor cells such as available from fine needle aspirates, circulating tumor cells or touch preparations.

Preliminary findings employing these response and correlative endpoint markers in the ongoing phase I trials at KCI will be presented.

Keywords: phase I trials, DNA-damage signaling, aminoflavone prodrug, PARP inhibition

296 Correlation of Cytokine and Angiogenic Factor (C/AF) Profiles With Clinical Outcomes Following Induction Chemotherapy in Head and Neck Squamous Cell Cancer (HN)

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Thirty-six cytokines and angiogenic factors (C/AFs) were measured in HN patients on a Phase II induction chemotherapy trial to investigate correlations between biomarker signatures and clinical outcomes. Previously untreated patients (n=47) received 6 weekly cycles of paclitaxel (135 mg/m^2), carboplatin (AUC 2), and cetuximab (400 mg/m^2 week 1; 250 mg/m^2 weeks 2-6) followed by definitive local therapy. Eligible patients were stage T0-4, N2b/c/3, M0; PS 0-1; with any HN primary site. There were 12 complete and 34 partial responses (MS Kies, et al., 2006 ASCO Annual Meeting) to induction therapy. Serum from 32 patients at baseline and at the end of chemotherapy was analyzed in duplicate by multiplex bead assay. IGF-1 was quantified by ELISA. Factors that did not meet predetermined criteria were excluded or retested. Statistical analysis was performed to compare biomarkers between patients and time points.

After a minimum follow-up of 2 years, 6 patients have recurred. Although patients with recurrence had higher T-stage, there was no statistical difference in nodal stage or EGFR status. Eleven C/AFs were statistically ($p \leq 0.05$) higher at baseline in patients who later recurred, and three of these factors (IL-6, VEGF, and eotaxin) remained elevated at the end of treatment. Recurrence was also associated with rising levels of Gro-alpha, MCP-1, and MCP-3 over the course of treatment. Unsupervised hierarchical clustering of baseline levels for all 36 C/AFs identified two distinct patient subgroups. One subgroup (n=19) included all recurrences and was characterized by high levels of hypoxia-associated factors (ex., VEGF, IL-4, PDGF, IFN-) . Log-rank comparison of progression-free survival between subgroups had a *p*-value of 0.029, despite there being similar distribution of disease stages in each subgroup.

In conclusion, C/AF profiling identified differences in serum proteins in HN patients with disease progression. These preliminary data suggest that C/AF is superior to immunohistochemical staining in identifying markers of hypoxia and in its prognostic value. C/AF signatures will be validated in an independent patient cohort.

Keywords: angiogenic factors, serum profiling, hypoxia

297 Level of Hypoxia-Inducible Factors HIF1α and HIF2α in Tumors Predicts Response to Sunitinib in Renal Cell Carcinoma Patients

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Background: Up to 40% of metastatic renal cell carcinoma (RCC) patients achieve an objective [complete (CR) or partial (PR)] clinical response following sunitinib treatment (NEJM 2007:356:115-24). Identification of molecular markers that correlate with outcome may provide insight into mechanism of sunitinib action, as well as identify patients who may benefit.

Methods: Patients with metastatic clear cell RCC with tumors resected and stored at MSKCC prior to sunitinib treatment were eligible for this study. HIF1α and HIF2α levels were determined from lysates from fresh-frozen biopsy specimens following immunoblot analysis, and scored as : none, low, or high, HIF levels based on relative intensity of immunoblot staining (relative to controls). Best clinical response was assessed for patients to sunitinib per RECIST criteria. Biological and biochemical studies of Sunitinib response were carried out on a panel of 10 cultured RCC cell lines.

Results: There was a significant association between HIFα level found in tumors and best response to sunitinib treatment. Patients with tumors expressing high levels of HIF1α (p=0.003) or high level of HIF2α (p=0.001) were more likely to achieve a favorable objective clinical response (CR or PR) to sunitinib when compared to patients with tumors containing low or no HIFα levels. For example, 13 of the 17 patients with high HIF1α had an objective response, while only 2 out of 11 patients with no detectable HIF1α had an objective response to sunitinib. Immunohistochemistry staining showed HIF1α is specifically present in the nucleus of cancer cells, and not within the adjacent stromal cells. In RCC cultured cells, sunitinib treatment resulted in cell death specifically in 5 of 10 RCC cell lines with high baseline HIF1α and HIF2α. In these cells, sunitinib treatment caused decreases in the levels of HIF1α and HIF2α by suppressing the translation of these proteins, thus resulting in cell death.

Conclusions: HIF1α and HIF2α may be important biomarkers for predicting clinical response to sunitinib therapy in RCC. Sunitinib functions to suppress levels of HIF1α and 2α, leading to cell death. These discoveries may lead to optimized treatment, particularly in patients with high HIF levels.

Keywords: clear cell renal cell carcinoma, metastatic, sunitinib response

298 GOG #210: A Molecular Staging Study of Endometrial Cancer – A Cooperative Group-Initiated Resource to Facilitate Translational Research

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High quality translational research (TR) is dependent on the standardized collection, maintenance, and distribution of well-annotated biospecimens. The GOG #210 protocol is a molecular and sugico-pathological staging study of endometrial cancer, including carcinosarcomas. The overall goal of this protocol is to improve outcome and/or quality of life for patients with endometrial cancer. This fundamental goal will be accomplished through the development of more accurate models of risk, identification of targets for therapeutic intervention, and utilization of individualized treatments based on molecular characteristics identified in tumor tissue, normal tissue, and/or readily accessible biologic fluids (e.g., serum and urine). The GOG #210 resource includes a repository of clinical specimens (i.e., fixed and frozen tumor and normal tissue, serum, and urine) annotated with detailed clinical, surgico-pathologic, follow-up, epidemiologic, and laboratory data from women who have surgically staged endometrial carcinoma. GOG #210 TR concepts and proposals utilizing conventional and high-throughput methods are reviewed by the GOG #210 Subcommittee and Committee on Experimental Medicine. Funded TR projects include gene expression risk profiling studies for lymph node metastasis and recurrence (DOD), FGFR2 mutation study (R21), and an epidemiology study (DCEG). Additionally, several submitted grants utilize GOG #210 resources, including two SPORE grants, three R01s, an R21, and a Foundation grant. These studies encompass populations of women at risk, with early or late disease, with rare tumors, and/or who are minorities/underserved. The GOG #210 resource was designed to foster the development of novel biomarkers of endometrial cancer risk, diagnosis, and prognosis, expand our understanding of endometrial cancer, and ultimately translate to improved clinical management and outcome.

Keywords: endometrial cancer, genomic analysis, grant support

299 Defining Genetic Subgroups of Melanoma and Determining Optimal Targeted Therapy: Parallel Preclinical Studies and Clinical Trials

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The identification of somatic genetic changes that activate signal transduction pathways at distinct points has allowed for the delineation of genetic subsets of tumors for which specific targeted therapies can be developed. Based on mutual exclusitivity of activating mutations in the MAPK pathway, melanomas can be stratified into three groups: *BRAF* (60%), *NRAS* (15%) and *KIT* (3%). However, additional mutations are known to occur in the PI3K and p53/Rb pathways which will likely influence responsiveness to MAPK pathway targeted therapy. We currently characterize these mutations prospectively for patients with metastatic melanoma for inclusion into clinical trials. We are evaluating the first selective BRAF inhibitor (PLX4032) with preclinical activity only in *BRAF*-V600E mutated tumors in a phase I/II trial; a less selective BRAF inhibitor (RAF-265) which demonstrates activity against *BRAF* and *NRAS* mutant cell lines in a separate phase I/II trial; and the c-kit inhibitor, imatinib, in a phase II trial for patients with *KIT* mutations.

While conducting the clinical trials with PLX4032 and RAF-265 we are interested in identifying determinants of therapeutic resistance *in vitro* to BRAF-inhibitors in melanomas that carry the common BRAF V600E mutation. We identified a set of metastatic melanomas and melanoma cell lines, which harbored either concurrent *BRAF* V600E and*CDK4* mutations or concurrent *BRAF* V600E and cyclin D1 (*CCND1*) amplification. We found that cell lines with a *CDK4* mutation alone did not have increased resistance to a BRAF-inhibitor, whereas a cell line with a *CDK* mutation and *CCND1* amplification did. We overexpressed CCND1 alone and in the presence of CDK4 in a drug sensitive melanoma line. CCND1 overexpression alone increased resistance, which was enhanced with concurrent overexpressed of CDK4. Increased levels of cyclin D1, resulting from genomic amplification, may contribute to the BRAF-inhibitor resistance of *BRAF*-V600E mutated melanomas. Secondly, we were interested in determining whether non-V600E *BRAF* mutated melanomas responded differently to therapies than V600E melanomas. We identified two additional subgroups with genetic activation of the MAPK pathway for which distinct therapeutic strategies appear appropriate. Melanoma lines with non-V600E mutations in BRAF (G469E, D549V) are highly resistant to MEK inhibition; however they were sensitive to the CRAF inhibitor sorafenib; unlike *BRAF* V600E cell lines.

Prior studies have shown low-activity mutants of *BRAF* signal via CRAF. Sorafenib down-regulated CRAF targets, suggesting that sorafenib may be ideally suited for this small subset of melanomas. Lastly, we wanted to study melanomas without activating mutations in *BRAF* or *NRAS*, as these constitute a substantial group of tumors. We used a genomic strategy to identify a group of melanomas and melanoma cell lines with co-amplification of *CDK4* and *KIT*. Pharmacological studies showed they were resistant to BRAF inhibitors but sensitive to imatinib in both *in vitro* and *in vivo* melanoma models. This may be a sub-population, in addition to those with KIT mutations, for which KIT inhibitors could be used. Based on our studies, we can suggest optimal therapies for the two novel sub-groups of melanomas, sorafenib (or other CRAF inhibitors) in non-V600E BRAF mutated and imatinib (or other KIT inhibitors) *KIT*/*CDK4* co-amplified melanomas. In addition, we have demonstrated in patients with *BRAF* V600E mutations, additional genotyping will need to be done in order to identify factors that confer resistance to Raf inhibitors. In particular concurrent *CCND1* amplification appear likely to confer resistance to BRAF-inhibitors. If validated, subsequent phase II trials would focus on patients whose melanoma harbor *BRAF* V600E, but not *CCND1* amplification. These studies strongly support the genetic classification of melanomas should be done prior to treatment and demonstrate how they can be used to guide therapeutic selection.

Keywords: melanoma, somatic mutations, kinase inhibitors

300 Discriminatory Accuracy From Single Nucleotide Polymorphisms (SNPs) in Models to Predict Breast Cancer Risk

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Models such as the National Cancer Institute's Breast Cancer Risk Assessment Tool (BCRAT), also known as "Gail model 2," are used to estimate a woman's risk of developing breast cancer over a defined age interval. The data for BCRAT include age at first live birth, age at menarche, number of previous breast biopsies, and number of mother or sisters with breast cancer. BCRAT has been criticized for its limited ability to discriminate women who will develop breast cancer from women who will not. This discriminatory accuracy is usually measured as the area under the receiver operation characteristic curve (AUC). In this study, I estimated how much the AUC of BCRAT could be increased by adding information from seven SNPs that have been shown to be associated with breast cancer.

I used published estimates of odds ratios and allele frequencies for seven SNPs that were discovered in two recent genome-wide association studies (GWASs) and in one candidate gene study. Assuming the odds ratios act on risk multiplicatively, I estimated the AUC for four risk models based on: the 7 SNPs; 14 such SNPs; BCRAT; and BCRAT plus the 7 SNPs. The AUC values for these four models were respectively 0.574, 0.604, 0.607, and 0.632. Thus BCRAT had more discriminatory accuracy than the 7-SNP model and than a hypothetical 14-SNP model, but the seven SNPs add modestly to the discriminatory accuracy of the BCRAT.

From work by Pharoah and colleagues, I estimated that a polygenic component of familial risk, conferred by SNPs, could yield an AUC of 0.800. However, to attain this goal, about 300 breast cancer associated SNPs would be needed. To discover these SNPs, most of which have very small odds ratios, would require huge numbers of cases and controls in GWASs.

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Keywords: breast cancer risk model, SNPs, discriminatory accuracy

301 The Prognostic Significance of Ki67 Expression in Stage I/II Invasive Cutaneous Melanoma

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Tumor cell proliferation is a key characteristic of stepwise tumor progression. The first step in melanoma progression is the radial growth phase (RGP) of the primary lesion. This may be in situ or invasive (the latter is characterized by atypical melanocytes that have entered the dermis and that have markers of diminished proliferation, i.e., no dermal mitoses and no tumor nests larger than those in the epidermis). In the next step, vertical growth phase (VGP), proliferation is again apparent, as reflected in dermal mitoses ("mitogenic VGP") and/or tumor cell nests larger than any epidermal nest. An alternative to evaluating mitoses to characterize cellular division is to detect expression of Ki67 protein. Since Ki67 is expressed in all phases of the cell cycle except G_0, it has the potential to be a more sensitive biomarker for cellular proliferation than mitoses. In a large cohort of patients with "thin" melanomas (≤1 mm in Breslow thickness) we previously demonstrated that Ki67 expression was an independent prognostic factor for development of metastases and illustrated its clinical application as a complement to the more established biomarker of dermal mitoses.

Here we examined the receiver operating characteristic (ROC) curves and the prognostic value of Ki67 expression for ten-year melanoma-specific death (MSD) in 600 patients with melanomas of all thicknesses (stages I/II) who had 10 to 35 years of follow-up. Ki67 expression was assessed by immunohistochemistry using the monoclonal antibody MIB-1. The percent of positive melanoma cells in the dermis was evaluated by two readers. The optimal cut point was selected maximizing sensitivity and specificity with a confidence interval obtained by bootstrapping. Univariate and multivariate logistic regression models and prognostic trees for ten-year MSD were used to evaluate the prognostic significance of Ki67 expression.

There was a significant association between Ki67 expression and ten-year MSD: the odds ratio (OR) was 1.07 (95% CI: 1.06-1.09) and the area under the ROC was 0.76. The optimal cut point for Ki67 expression was 25% (95% CI: 21%–32%). The sensitivity and specificity using the cut point ≥ 25% were 58% and 87%, respectively. The odds of ten-year MSD with high Ki67 expression (≥ 25%) was 3-fold higher than the odds with low Ki67 expression (< 25%) after controlling for thickness, ulceration, mitotic rate, tumor infiltrating lymphocytes, gender, and anatomic site. The prognostic tree identified mitogenicity as the best predictor of ten-year MSD for patients with thin melanomas (≤ 1 mm): the rates for non-mitogenic and mitogenic thin melanomas were 1.7% and 13%, respectively. High Ki67 expression (≥ 25%) was the best predictor for patients with thicker melanomas (> 1mm): for these patients, rates for high (≥ 25%) and low (< 25%) Ki67 expression were 25.2% and 53.7%, respectively.

In the light of recent studies identifying a role for mitotic rate in prognosis and in prediction for sentinel lymph node biopsies, these data suggest that Ki67 expression be considered as an adjunct biomarker to evaluating mitoses, especially in thicker melanomas. Our findings need to be replicated in future prospective studies of clinical outcomes including the prediction of nodal status.

Keywords: Ki67, mitoses, MIB-1

302 A Four Gene Expression Ratio Test Prospectively Predicts Survival in Patients Undergoing Surgery for Mesothelioma

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Background: Malignant pleural mesothelioma (MPM) is a rapidly fatal neoplasm with few effective treatments, one being cytoreductive surgery. We previously described a predictive test, based on ratios of gene expression levels, for the outcome of MPM patients undergoing surgery. Here we determined the accuracy, repeatability, and reproducibility of this test in prospectively consented patients.

Methods: Tumor specimens linked to clinical data were prospectively obtained from 120 consecutive patients undergoing surgery for MPM at a single institution. A 4-gene predictive test was used to assign patients to either a good- or poor-outcome group. Its predictive utility was evaluated using standard methods for survival analysis, including multivariate modeling to assess predictive value of the gene ratio test independent of traditional prognostic variables. The technical robustness of the test was also determined by using multiple specimens per patient, biopsy techniques, and performance sites.

Results: The test predicted overall survival and cancer specific survival in a statistically significant manner in both univariate and multivariate analyses. It also proved highly reproducible and repeatable over a large range of tumor cell content for specimens obtained by either debulking surgery or minimally invasive pleural biopsy. A predictive scheme to identify good surgical candidates which incorporates this test with other known prognostic factors has been developed.

Conclusions: This MPM predictive test is an appropriate adjunct to traditional prognostic variables including stage and histology. It is likely to prove useful in stratifying patients with MPM for treatment algorithms, in order to identify patients who will benefit from surgery.

Keywords: predictive test, RNA expression, mesothelioma

303 DNA Methylation, Gene Expression, and Genetic Background as Markers for Advanced Melanoma and Responsiveness to 5-Aza-2'-Deoxy-Cytidine Induced Growth Arrest

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Aberrant changes in gene activity due to chromatin remodeling involve methylation/demethylation of cytosine at cytosine-guanine (CpG) pair rich islands in promoter regions and post-transcriptional modifications (acetylation/methylation) of histones. Aberrant gain or loss of DNA methylation causes altered expression of genes associated with the malignant phenotype and can be used as a tumor marker. Furthermore, the reversible nature of epigenetic changes in chromatin is the rationale for clinical trials with DNA demethylation agents such as 5-Aza-2'-deoxy-cytidine (5-Aza-CdR, also known as decitabine), or its analogue 5-azacytidine. However, patients' responses are variable and there is a need for molecular markers that can predict and/or monitor the efficacy of therapy. There is also a need to identify possible targets that can synergize with 5-Aza-CdR, especially since combination therapies have shown to enhance 5-Aza-CdR's anti-cancer effect.

Our goal is to identify genetic and epigenetic markers, as well as gene expression signatures associated with the development of melanoma, resistance and sensitivity to 5-Aza-CdR, and the identification of agents that can act in synergy with the drug. Toward these goals, we monitored the sensitivity of 8 patient-derived tumor cells, most from short-term cultures, to 5-Aza-CdR employing cell proliferation and apoptotic assays. The melanoma cells were subjected to global differential gene expression analyses in response to low concentration (0.2 μM) of 5-Aza-CdR employing NimbleGen whole genome expression arrays. We also performed MeDIP (Methylated DNA Immuno-Precipitation) assays to capture and contrast whole genome expression and methylation status in melanoma and normal melanocytes. Further, we sequenced melanoma cells for known melanoma mutations.

In agreement with the clinical experience, our examination revealed that only a subset of 5-Aza-CdR treated cells exhibited signs of growth arrest and apoptosis. Whole-genome differential gene expression, as well as selective protein analyses, ruled out the involvement of genes and proteins involved in the DNA damage response and p53 induction. The finding is consistent with the fact that we used a low concentration (0.2 μM) of 5-Aza--CdR, which acts by incorporating into DNA and downregulation of DNMT. On a global scale, gene enrichment analysis using Gene Ontology (GO) revealed that 5-Aza-CdR caused differential gene expression of genes associated with the extracellular region, response to wounding, response to external stimulus, protease inhibitor activity, and genes associated with acute inflammatory and immune responses. Experimentally, we have identified several pathways that can lead to growth arrest in sensitive cells: induction of $p21^{Cip1}$, activation of TGFβ pathway genes, and activation of signaling modulators such as PTPN6 and IGFBP5. Furthermore, analyses based on known genetic mutations ruled out the involvement of BRAF mutation in 5-Aza-CdR responsiveness, but underscored the role of activated β-catenin in contributing to 5-Aza-CdR resistance. Based on proteasomal degradation of key proteins, we demonstrated beneficial synergy between 5-Aza-CdR and the inhibitors MG132 and Velcade in 5-Aza-CdR resistant melanoma cell lines. MeDIP analyses followed by bisulfite-modified DNA sequencing revealed a set of genes that are suppressed by promoter methylation in advanced, but not primary melanoma cells, that are slightly re-activated by 5-Aza-CdR. We hypothesized that the components analyzed here could be used as biomarkers for advanced melanoma, patient's selection, monitoring treatment, and devising better synergistic therapy to this DNA modifying agent.

Keywords: MeDIP, growth-arrest pathways, proteasomal degradation

304 Identification of Molecular Subtypes of Glioblastoma

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Glioblastoma multiforme (GBM) is the most prevalent form of brain tumors and is highly aggressive, which results in poor patient outcomes. In order to identify possible subtypes of GBM, we expression profiled 202 GBM samples on three gene expression platforms. The data from these platforms were merged by factor analysis to create a unified gene expression measurement that was more stable than any single platform alone. Using only variably expressed genes, consensus clustering was performed and revealed four subtypes. Sample cluster/group validity was further determined by silhouette analysis, and only samples with positive silhouette widths were retained for further analysis as core samples that best represent each subtype (n=173). To create class predictors, we used a nearest centroids predictor, ClaNC, to identify 840 genes that could predict the four classes with 4.6% cross-validation error and a 1% training error. Application of the class predictor on an independent dataset (n=174, GBMs compiled from 3 datasets) showed the presence of these four subtypes, thus demonstrating their reproducibility.

While the subtypes were not prognostic for outcomes, correlations with clinical parameters were identified including associations with age, tumor cellularity and nuclear atypia. By definition, each subtype showed unique expression features; for example, the Proneural subtype has expression of genes that are expressed in neuronal stem cells such as the *SOX* genes and *CD133*. The Neuronal (Normal-like) subtype expresses genes required for differentiated neurons including genes involved in neuron projections and synaptic processes. The EGFR subtype was defined by very high gene expression of *EGFR* and is amplified at the EGFR locus in almost all cases. The Mesenchymal subtype has high expression of genes including the tumor necrosis superfamily and downstream NF-kB pathway components such as collagens, cytokines, chemokines, integrins, and interleukins.

Interestingly, many of the most frequent mutations events identified in GBMs were significantly associated with subtype. Three of the top eight mutated genes identified in GBMs showed associations with subtypes including *TP53*, *NF1*, and *PIK3CA*. In addition, many tumor DNA copy number events were associated with expression subtypes including the deletions of 17q11.2, which contains *NF1*, and 13q14, which contains *RB1*, being frequent in the Mesenchymal subtype. By identifying molecular subtypes through gene expression and identifying correlations with additional data such as copy number and mutation, we have gained insights into GBM biology, which should help direct treatment options.

Keywords: glioblastoma, subtypes, gene expression

305 Sensitive Location and Characterization of Circulating Tumor Cells for Improving Therapy Selection and Monitoring Treatment Efficacy

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For metastatic disease, biomarker profiling of distant metastases is done only when feasible because biopsy of metastases is invasive and associated with potential morbidity without proven benefit. So although biomarker expression may differ in distant metastases, treatment with targeted therapies is almost always based on biomarker targets derived from a patient's primary breast tumor, usually excised years before development of metastatic disease.

Circulating tumor cells (CTCs) in the blood have been linked to disease outcome in patients with metastatic breast cancer. An FDA-approved kit that counts CTCs has been shown to be superior to conventional imaging for predicting progression-free and overall survival. The sensitivity of this methodology is subject to expression levels of its immunomagnetic target, EpCAM, and is only able to measure one biomarker beyond those needed for CTC identification. The methodology is not used to guide choice of specific drug therapies.

We have developed a sensitive CTC detection tool using Fiber Array Scanning Technology (FAST) that can rapidly locate CTCs on a substrate and uses abundant cytokeratins not EpCAM as targets. We have developed a protocol that enables testing for protein expression of 5 markers in addition to the ones needed for CTC identification. We are currently testing the use of multimarker characterization and CTC enumeration for improving therapy selection and monitoring treatment efficacy in several retrospective studies.

Rationale: The modality being developed is "the use of FAST to locate CTCs for characterization and numeration to improve treatment selection and monitor efficacy". FAST is an image based assessment tool, and its basic technical characteristics have already been validated/credentialed. This fits the "creation of modality" step best since it is being tested in several retrospective cohorts, although it is noted that it represents two different though related modalities – one for improvement of treatment selection and the second for monitoring efficacy.

Keywords: circulating tumor cell, biomarker, enrichment

306 Normalization of the Tumor Microenvironment for Therapy: Bench to Bedside and Back

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In the past 8 years, our Program Project team has used a systems approach to overcome the vascular and interstitial barriers impeding delivery and efficacy of molecular medicine. Our most exciting and clinically relevant finding is that when the balance of pro- and anti-angiogenic molecules is partially restored in tumors by VEGF blockade, the aberrant structure and function of the vasculature and interstitial matrix transiently become closer to that of normal tissue. Referred to as vascular normalization, this concept has generated a new paradigm in the field of anti-angiogenic therapy and provided an explanation of how antiangiogenic therapy enhances chemo- and radiation therapy. We have tested this concept in rectal carcinoma patients receiving the anti-VEGF antibody bevacizumab (Avastin) with chemo- and radiation therapies, and our clinical findings mirrored those made in our transplanted tumor models in Project 1 (Willett et al., *Nat Med* 2004). Several independent laboratories have published data in support of our findings on vascular normalization. Most importantly, our work has spawned multidisciplinary clinical studies of antibodies or tyrosine kinase inhibitors that target VEGF and/or PDGF pathways for glioblastoma, sarcomas, breast, liver, ovarian, head and neck patients at the MGH.

In our Program Project, we address critical issues in the clinical translation of anti-angiogenic therapy of tumors. The overall theme is to characterize tumor response to antiangiogenic agents—currently in clinical testing—that differentially target the VEGF and PDGF signaling pathways. Understanding the effect of each agent on vascular normalization will allow the design of specific regimens for the combination of cytotoxic therapies with each of these antiangiogenic agents. In Project 1, the goal is to improve these treatments by manipulating perivascular cell recruitment. We found that normalization of tumor vasculature by improved perivascular cell support makes it more efficient for oxygen delivery and thus, enhances tumor response to radiotherapy (Kashiwagi et al., *Nat Med* 2008). In addition, we aim to establish a "normalization index" for each antiangiogenic agent, determine drug delivery and tumor response and complement studies in Project 1 by evaluating surrogate markers of biological response (circulating proteins and progenitor cells) in Project 2. We established that normalization of vascular function can decrease edema in brain tumor models, prolonging survival (Kamoun et al., submitted). Finally, we are studying the role of cytokines and proteases in the permeabilization of the collagen matrix in tumors, with the aim of using agents that modify extracellular matrix to improve gene therapy in Project 3. We discovered that degradation of fibrillar collagen increases the distribution and efficacy of oncolytic viruses (McKee et al., *Cancer Res* 2006 and Mok et al., *Cancer Res* 2007). Each Project relies upon unique *in vitro* and *in vivo* models, powerful intravital techniques, innovative imaging technologies, mathematical modeling and statistical support (Core A); cutting-edge molecular, cellular, and histological expertise (Core B); superb surgical and animal support (Core C); and administrative support (Core D). We are testing the preclinical studies in blood and tissue obtained from clinical trials. With this infrastructure, and the help of our clinical collaborators, we intend to develop optimal strategies for cancer therapy and translate these scientific discoveries in clinical studies.

Keywords: normalization, tumor microenvironment, drug distribution

307 Urine PGE2 Metabolite as a Possible Biomarker in Patients (pts) With Non-Small Cell Lung Cancer (NSCLC)

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Upregulation of cyclooxygenase-2 (COX-2), increased PGE synthase and/or downregulation of 15-prostaglandin dehydrogenase (15-PGDH) are frequently observed in NSCLC. These alterations in the eicosanoid pathway promote high intratumoral/systemic prostaglandin E2 (PGE2) levels that contribute to the development and growth of NSCLC. Efforts to reduce PGE2 levels, e.g. COX-2 inhibition (COX-2i), could prove beneficial in the treatment of NSCLC. To date studies of COX-2i in human NSCLC have yielded mixed results. We hypothesize these discordant results are due to two possible flaws in prior approaches: lack of validated biomarkers for selecting pts likely to benefit for COX-2i and pleiotropic effects of the available inhibitors on multiple, previously unmeasured arachidonic acid metabolites. Measurement of excreted urinary metabolites using mass spectrometry is the most accurate index of endogenous eicosanoid production in humans. We developed an assay to measure the metabolite of PGE2 (11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid; PGE-M) to assess adequacy of COX-2i in human trials.

In preliminary studies in pts with either resectable or recurrent NSCLC, celecoxib (C) (400 mg BID x 5-7 days) decreased intratumoral PGE2 levels and resulted in a concomitant decline in PGE-M. In a subsequent phase II trial 54 unselected pts with recurrent NSCLC received C with docetaxel (Doc) (preclinical studies demonstrated additive cytotoxicity); median survival (MST) = 6 mo (95% CI, 4.7-8.2; range, 0.6-38.3). In a multivariate model accounting for sex, smoking history, and histology, we observed a strong association with change in PGE-M levels and survival. Pts with the greatest proportional PGE-M decline following C had a ≈50% decrease in the relative risk of death (MST = 14.8 mo; 1-yr = 36%). By contrast pts whose PGE-M levels rose 50% after C had a MST = 5 mo and no 1-yr survivors ($P<0.001$). These data support PGE-M as a biomarker of intratumoral COX-2 activity and PGE2 production. The data also suggest COX-2i may benefit selected NSCLC tumors characterized as "COX dependent" (defined as a ≥70% decline in PGE-M levels pre- and post-C). We have initiated a phase II trial combining C with Doc or pemetrexed in pts with recurrent, "COX dependent" NSCLC as determined by change in urine PGE-M following a brief course of C. The primary endpoint is overall survival (a 50% increase in MST).

Secondary objectives include assessment of response rates and times to progression, effect on prostacyclin and thromboxane production as well as correlation of PGE-M changes with intratumoral expression of COX-2 and 15-PGDH. In addition, there remain concerns about the cardiac toxicity associated with selective COX-2i as well as the possibility that not every NSCLC patient will derive benefit from COX-2i due to other changes in the eicosanoid pathway listed above. Prostaglandin E2 exerts its cellular effects by binding to its cognate receptors (EP1-4). Examination of NSCLC cell lines show increased EP4 expression in 11/18 lines, and in preclinical studies, we have shown that a specific antagonist of EP4 (ONO-AE3-208) has a profound inhibitory effect on lung cancer metastasis. These data suggest that specific inhibition of EP4 could afford beneficial therapeutic effects. Currently, we are conducting orthotopic xenograft mouse models to determine the role of newly designed EP4 antagonists (MF-191) in inhibition of primary tumor growth and metastasis, and we are examining the molecular mechanisms involved in this process.

Keywords: PGE2, cyclooxygenase-2, non-small cell lung cancer

308 Validation of Genomic Targets in Melanoma

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Recent cDNA microarray analyses of nevi, primary and metastatic melanomas performed in our laboratory have identified a large number of genes whose level of expression can be used to distinguish between known stages in the tumor progression cascade of melanoma. To date, we have confirmed the utility of several of the genes suggested by that analysis as novel biomarkers for melanoma. To accomplish this, we created a tissue microarray (TMA) of over 350 primary melanoma specimens with either two years of follow up, first relapse, or undergoing sentinel lymph node (SLN) biopsy. Among the genes overexpressed in our analyses were NCOA3 (nuclear receptor coactivator 3), SPP1 (osteopontin), and RGS1 (regulator of G signaling protein 1). We assessed the prognostic significance of NCOA3, SPP1, and RGS1 expression using immunohistochemical analysis of the TMA. For each of the markers, marker overexpression was significantly predictive of SLN metastasis. Kaplan-Meier analysis demonstrated a significant association between marker overexpression and reduced relapse-free (RFS) and disease-specific (DSS) survival. Logistic regression analysis revealed marker expression to be an independent predictor of SLN status. Multivariate Cox regression analysis showed that marker expression independently predictive of DSS. A multi-marker index combining the expression level of the three markers was developed for its ability to predict various outcomes associated with melanoma. Increasing multi-marker index scores were significantly predictive of SLN metastasis and reduced RFS, DMFS (distant metastasis-free survival), and DSS. Multivariate regression analyses revealed multi-marker expression scores to be an independent predictor of SLN status, RFS, DMFS, and DSS. The multi-marker index was the most powerful factor predicting DSS, and remained so even after including SLN status in the multivariate model. The multi-marker index was independent of AJCC stage in predicting DSS. Systemic ribozyme and siRNA-based targeting of several of the markers identified by the gene expression profiling analyses suppressed the metastatic progression of melanoma in murine models. These studies have identified novel biomarkers for melanoma and novel targets for therapy of melanoma metastasis.

Keywords: melanoma, biomarker, tissue microarray

309 Biomarkers to Predict Response to Organ Sparing Therapy in Oropharynx Cancer

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Purpose: To identify markers of response to therapy and outcome in oropharynx cancer.

Patients and Methods: Patient variables, including smoking history, age, gender, primary site, stage and nodal status as well as biomarkers in pretreatment biopsies, including HPV copy number, EGFR, p16, BCLXL and p53 expression and p53 mutation were examined and assessed for association with high risk HPV, response to therapy, and survival.

Results: HPV copy number and p16 expression were both significantly associated with response to treatment with induction chemotherapy, response to concurrent chemoradiation, and overall and-disease specific survival. In contrast, EGFR expression was inversely associated with response to induction chemotherapy, chemo/RT, overall survival, and disease-specific survival, but directly associated with current smoking, female gender, and lower HPV titer. As combined markers, lower HPV copy number with high EGFR expression or combined low p53 and high BCLXL expression were each associated with poorer overall survival and disease-specific survival.

Conclusion: Low EGFR and high p16 (or higher HPV titer) expression are markers of good response to organ sparing therapy and outcome, whereas high EGFR expression, combined low p53/high BCLXL expression, female gender, and smoking are associated with poor outcome. We plan to use pretreatment biomarkers and patient characteristics to select patients for appropriately targeted individualized therapy to improve overall responses and increase survival and quality of life.

Keywords: oropharyngeal cancer, biomarkers, HPV-16

310 Association of VEGF Polymorphisms With Primary Melanoma Tumor Thickness and Ulceration

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Background: VEGF may play a role in the regulation of angiogenesis in melanoma tumors. We therefore investigated potential associations between VEGF polymorphisms and primary tumor histopathology in a large cohort of melanoma patients.

Methods: VEGF (-2578 A/C, -1154 A/G, -634 C/G, +936 C/T) single-nucleotide polymorphisms (SNPs) were determined by analysis of genomic DNA from melanoma patients. Clinical data, including primary tumor histopathology, were obtained from a prospectively maintained patient database. Relationships between VEGF SNPs and tumor histopathology were evaluated by T-test, ANOVA, chi-square, and Fisher exact test as indicated. Haplotype frequencies were estimated using the EM algorithm method, and additive linear regression was used to determine associations between VEGF haplotypes and tumor histopathology.

Results: 778 patients underwent VEGF SNP analysis, including 620 patients who presented with localized disease (stage I-II), 145 with regional disease (stage III), and 13 with distant disease (stage IV). The median primary tumor thickness was 1.1 mm and 22% of tumors were ulcerated. Patients with the VEGF -2578 C polymorphism had thinner tumors compared to patients without this polymorphism (median 1.19 vs. 1.30 mm, P=0.005), while VEGF +936 T was associated with thicker tumors (1.37 vs. 1.10 mm, P=0.005). Ulceration was also more common in patients with +936 T (28% vs. 19%, P=0.03). Furthermore, copy number analysis demonstrated a dose effect for these associations (Table). Finally, haplotype analysis confirmed the association of the VEGF -2578/-1154/-634/+936 haplotypes AGGT (OR 3.80, P=0.001) and AAGT (OR 1.75, P=0.012) with thicker tumors, and the AAGT haplotype with ulceration (OR 6.05, P=0.007).

Conclusions: VEGF polymorphisms are associated with the primary melanoma histopathologic prognostic factors of tumor thickness and ulceration. These results suggest that regulation of angiogenesis by VEGF polymorphisms may help determine melanoma tumor thickness and ulceration, and that VEGF could be an effective target for adjuvant therapy and prevention of melanoma.

Association of VEGF SNP Copy Number With Primary Melanoma Histopathology

VEGF polymorphism	Number of copies	Tumor thickness (median, mm)	P	% Ulcerated	P
-2578 C	0	1.30	**0.019**	24.3	NS
	1	1.20		21.8	
	2	1.07		17.8	
+936 T	0	1.10	**0.015**	19.5	**0.024**
	1	1.31		27.0	
	2	1.75		45.4	

Keywords: melanoma, VEGF, angiogenesis

311 Global Proteomic Analysis of HNSCC Identifies Prognostic Markers

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Objective: The overall goal of this study is to develop independent and significant predictive measures of tumor behavior for head and neck squamous cell carcinoma (HNSCC).

Methods: In our systems biology approach to study HNSCC, we extract tumors with TRIzol® to isolate DNA, RNA and protein from the same piece of tissue for both independent and integrated analyses. We have developed and validated a method for detecting biologically significant differences in protein expression in two head and neck tumor cell lines (Sudha et al. Laboratory Investigation, 2007). The feasibility study demonstrated that the procedure is highly reliable for identifying peptides that distinguish biological variability among samples, and can therefore be used to identify potential prognostic biomarkers for disease.

Results: In the present study 48 primary HNSCC specimens obtained from oral cavity, oropharynx and larynx/hypopharynx have been analyzed. Global proteomic analysis from these experiments is being used as a "training set" to discriminate/classify HNSCC with different clinical outcomes. When the entire data set was analyzed irrespective of tumor site, we identified 15 peptides that correlate with clinical outcome. When the three sites were analyzed separately, we identified 57 peptides that correlate with clinical outcome. Data analysis by anatomic site of the HNSCC has produced more definitive results both for global RNA and global proteomic data sets. Validation by tissue microarray for individual proteins and analyses on additional samples are being performed.

Conclusion: The results strongly suggest that a small number of peptides can be used to develop a predictive model that can discriminate disease severity at initial diagnosis as well as clinical outcome prospectively. RNA expression profiling of primary HNSCC specimens have also been performed. We will integrate the proteomic data with the RNA expression data to identify the best potential prognostic biomarkers to develop clinical diagnostics.

In the future, we will again refine and validate the predictive importance of peptide discriminators by analyzing an additional 87 HNSCC samples part of which serves as a "test set" with respect to tumor behavior and clinical outcome as well as obtain more precise estimates of effect.

Keywords: head and neck squamous cell carcinoma, global proteomics, prognostic markers

312 SH2 Profiling: A Sensitive, High-Throughput, Quantitative Method for Tumor Classification Based on Phosphotyrosine Signaling State

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Tyrosine phosphorylation controls many aspects of tumor cell behavior including proliferation, differentiation, adhesion and motility, and deregulated tyrosine phosphorylation plays a causative role for some tumors. Therefore the ability to monitor and profile the activity of tyrosine phosphorylation mediated signaling pathways would provide a biologically meaningful means of classifying tumors of otherwise similar pathological grade and stage. Such classification may provide clinically useful prognostic information such as predicting risk of recurrence or response to specific therapies.

We have developed a sensitive, quantitative, high-throughput method to quantitatively profile the phosphotyrosine signaling state of a cell sample, termed SH2 profiling. This method is based on the binding of a battery of Src Homology 2 (SH2) domain probes to the sample. Quantitative SH2 profiling data can then be used as the basis for classification, using either unsupervised or supervised algorithms. Furthermore, SH2 profiling can reveal important pathway information (what specific signal transduction pathways are activated in a particular tumor) and lead to identification of novel biomarkers (what specific phosphoproteins correlate with particular clinical outcomes).

We have generated pilot and feasibility data showing that SH2 profiling can be used to classify various types of human tumors and tumor cell lines, including chronic myelogenous leukemia, breast carcinoma, and non-small cell lung carcinoma (NSCLC). In the case of breast cancers, clustering based on SH2 profiling correlated with Her2/Neu amplification. For NSCLC, clustering based on SH2 profile correlated with mutation of EGF receptor and susceptibility to small-molecule EGF receptor inhibitors. These data indicate that SH2 profiling can be used as the basis for tumor classification, and provide a rich source of information on signaling pathway activity that could be correlated with clinical outcomes data in future studies. In particular, classification based on SH2 profiling is likely to be useful in predicting response to the many tyrosine kinase inhibitors now in use or under development.

Keywords: tyrosine phosphorylation, SH2 domain, tyrosine kinase inhibitors

313 Genetic Markers for Selecting High-risk Population With Oral Premalignancy for the Erlotinib Prevention of Oral Cancer (EPOC) Trial

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Introduction: Loss of heterozygosity (LOH) at chromosomal regions harboring tumor suppressor genes in oral leukoplakia lesions (OPL) has been associated with an increased risk of invasive oral cancer development in patients with this preneoplastic condition. LOH at these chromosomal regions, therefore, may serve as a genetic marker to evaluate oral cancer risk in patients with oral leukoplakia. We are incorporating this genetic marker as a criterion to select high-risk individuals to participate in an NCI-funded (P01 CA106451) clinical cancer prevention study, "Erlotinib Prevention of Oral Cancer (EPOC)."

Methods: EPOC is a double-blind, multi-center, randomized study of erlotinib versus placebo in patients with oral pre-malignancy. To be eligible for the trial, patients must meet one of the following two categories: 1) For patients with prior history of oral cancer, the resected surgical margins of tumors must contain LOH at 3p14 and/or 9p21; 2) for patients without oral cancer history but with oral intra-epithelial neoplasia, the lesions must contain LOH at 3p14 and/or 9p21 plus LOH at one other chromosomal region (i.e., 17p, 8p, 11p, 4q, or 13q). The genetic marker analyses are conducted centrally at MDACC within 7 business days of sample receipt. Eligible patients are then randomized (1:1) to placebo or erlotinib 150 mg/day for 1 year. The primary endpoint of the trial is oral cancer-free survival.

Current Status: The planned sample size is 150 patients. The study opened at M. D. Anderson in 11/06 and at The University of Chicago in 07/07. At this time, 227 patients have been screened and 151 patients (81 females, 70 males) have been enrolled (128 at MDACC and 23 at U. Chicago). Molecular eligibility was assessed in 138 patients (85 with prior oral cancer). Eighty-eight patients (57% and 73% of patients with and without prior or cancer, respectively) of the 138 patients met LOH eligibility criteria. Forty-eight of 88 eligible patients have been randomized so far. Two additional centers (Emory University, Atlanta, GA and Memorial Sloan-Kettering Cancer Center, New York, NY) are scheduled to be open for accrual by the end of 2008. **Conclusions**: As a translational component, LOH has been implemented as a genetic marker in a late-stage cancer prevention trial to enrich a population of patients with high oral cancer risk that would potentially most benefit from treatment. Centralized assessment of molecular eligibility within the context of the multi-institutional EPOC trial is feasible. Final results of the trial may prospectively confirm, for the first time, LOH as an oral cancer risk factor and could contribute to personalized risk assessments and chemoprevention strategies in the future.

Keywords: loss of heterozygosity, oral pre-malignancy, erlotinib.

314 Particle-Based Monoclonal Antibody Detection in Serum for Optimal Drug Dosing

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Monoclonal antibodies are used in the treatment of many cancers and proliferative diseases. Several approaches have been used to study the pharmacokinetics of these treatments. The target molecule can be made recombinantly for sandwich ELISA assays. However, it may not always be possible to generate the recognized portion of the target molecule and it is expensive and cumbersome to generate large amounts of recombinant protein. An alternate approach is to express the target molecule on a cell line by transfection, using flow cytometry to assess the binding of the desired mAb. This method has been used for alemtuzumab (anti-CD52), but is difficult to develop, requires skilled personnel to execute, and has limited sensitivity. Finally, ELISA assays have been developed that used antibodies specific for the therapeutic mAb. The antibodies used for this purpose are either anti-idiotypic or specific for residual non-human sequences of the therapeutic mAb, as was the case with alemtuzumab. However, each of these approaches is technically demanding and has limited sensitivity when used in biologic samples because of high background.

We selected peptide sequences recognized by alemtuzumab (anti-CD52) or rituximab (anti-CD20) from phage displayed peptide libraries. Synthetic biotinylated peptides were used in an enzyme linked immunoadsorbant assay (ELISA) and had a sensitivity of less than 0.05μg/ml in saline buffer, but the functional sensitivity in serum was limited to ~1ug/ml by the need to dilute samples to reduce background. Therefore, we developed a complementary immunoassay in which the peptides were synthesized on the surface of 10μm diameter tentagel beads. mAb binding was detected by fluorochrome labeled secondary antibodies via flow cytometry. There was negligible background signal on the beads, even in neat serum. The functional sensitivity using peptide-beads was less than 0.05μg/ml. The enhanced sensitivity of the bead based assay is ideal for detecting very low levels of the target antibody, while the conventional ELISA is sufficient when the target antibody concentrations present at concentrations of >1.0ug/ml. The process outlined here is generalizable to any mAb and could enable improved pharmacokinetic analysis during development and clinical use of these therapies. The clinical goal would be to regulate mAb dosage for each patient based on concentration in the blood instead of dosing based on body weigh in order to deliver an optimal dose to the patient and minimize the cost to the health care system.

Keywords: pharmacokinetics, personalized medicine, monoclonal antibodies

315 Early Clinical Trials Focused on Biomarkers

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Early clinical trials generally focus on drug development, using the classical paradigm of phase I testing to define the maximally tolerated dose, followed by phase II testing to measure the response rate at that dose. Although our phase I portfolio includes a number of classical phase I trials, we have conducted a number of studies that have focused on the development of biomarkers to predict toxicity and/or guide dosing of a particular agent. Two such studies have been completed and published, relating to irinotecan and erlotinib, respectively. A third study of sorafenib is ongoing.

The two published studies were prospectively designed to test the relationship between a candidate polymorphism and toxicity. The first study (Innocenti, J Clin Oncol, 2004), of the relationship of the *UGT1A1*28* promoter indel polymorphism to irinotecan-induced neutropenia, was the primary basis of a change to the Pfizer label for irinotecan (brand name, Camptosar), approved by the FDA in 2005. This label change included a warning regarding the increased risk of neutropenia in patients homozygous for this polymorphism, when the standard dose (350 mg/m^2) of irinotecan is administered.

The second study (Rudin, J Clin Oncol, 2008) was designed to test the relationship of polymorphisms in the *EGFR* promoter (Liu, Cancer Res, 2005) and intron 1 (Liu, Clin Cancer Res, 2003) to the skin and gastrointestinal toxicity of erlotinib. Variability in skin rash was best explained by a multivariate logistic regression model incorporating the trough erlotinib plasma concentration and the *EGFR* intron 1 polymorphism. Variability in diarrhea was associated with the two linked polymorphisms in the *EGFR* promoter, but not with erlotinib concentration. In addition, there appeared to be an association between erlotinib exposure and two new variants in the *ABCG2* promoter, suggesting that variability in expression of the ABCG2 transporter may have a major impact on the pharmacokinetics of this agent.

The third study of sorafenib is ongoing, and is designed to test the hypothesis that higher doses of sorafenib will lead to a greater effect on a potential biomarker for VEGF inhibition, ambulatory blood pressure. Previous studies from us and others have demonstrated that an increase in blood pressure is a mechanism-related toxicity of VEGF inhibitors. Thus, we have suggested that ambulatory blood pressure could be monitored to optimize dose and/or schedule of these agents. Our ongoing trial evaluates the impact of higher daily doses of sorafenib (400 mg bid vs. 400 mg tid vs. 600 mg bid) on the pharmacokinetics and pharmacodynamics of this agent. If a greater increase in blood pressure can be obtained with a higher dose, then this would support further randomized trials of higher doses vs. the standard 400 mg bid dose.

Keywords: pharmacogenetics, biomarker, blood pressure

316 Translational Research by the Cancer and Leukemia Group B (CALGB)

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The CALGB is an NCI-sponsored cooperative group that conducts clinical trials, correlative science studies and health outcomes research in all common adult solid tumors and hematological malignancies. The group is comprised of approximately 250 institutions and has 90-100 studies ongoing at any time that enroll about 3500 patients annually. In addition to its headquarters in Chicago and Statistical Center at Duke University, CALGB maintains 3 biospecimen repositories, an imaging core laboratory, a pharmacology core laboratory and 3 molecular reference laboratories that support the group's translational research activities. All biospecimens are obtained from patients enrolled on prospective clinical trials and all are fully annotated with respect to patient demographics and outcomes. CALGB biorepositories are all compliant with NCI guidelines for biospecimen banks and contain collections of paraffin embedded solid tumors, frozen lung cancer specimens with corresponding normal tissue, frozen leukemia cells and germline DNA obtained for pharmacogenetic studies. Recent CALGB correlative science studies have demonstrated that adjuvant paclitaxel is effective only in women with ER negative or Her2 positive early stage breast cancer: that COX-2 overexpression is associated with poor survival in patients with advanced NSCLC but is predictive of benefit from the addition of celecoxib to chemotherapy; that DNA mismatch repair deficiency in the primary colon tumor may predict benefit from irinotecan-containing adjuvant chemotherapy; that high plasma or urine VEGF levels are associated with poor prognosis in men with castrate resistant metastatic prostate cancer; that KIT gene mutations confer a higher risk of relapse and decreased overall survival in AML patients with inv(16); and that AML patients with RAS mutations benefit most from high dose cytarabine. Ongoing and planned CALGB studies will screen for RAS mutation to determine eligibility for cetuximab-based treatment for colorectal cancer; assess FLT3 mutations in AML to determine patient eligibility for a study that assesses a novel FLT3 inhibitor; prospectively study the utility of celecoxib in NSCLC patients with COX-2 overexpressing tumors and use gene expression profiling to assign risk of relapse to patients with early stage NSCLC or to determine treatment assignment to those with late stage disease. Studies in Hodgkin lymphoma and esophageal cancer will use risk-adapted treatment approaches based on the results of PET scans performed early in the course of treatment. The translational research studies of CALGB are transforming our approach to cancer treatment by enabling individualized treatment based on better understanding of cancer biology.

Keywords: biospecimens, breast cancer, leukemia

317 Comparison of Risk Stratification by the Combined HOXB13/IL17BR and Molecular Grade Indices Versus OncotypeDX in Early Stage ER+ Breast Cancer Patients From Multiple Institutions

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Treatment selection for early stage ER+ breast cancer patients remains a significant challenge and requires careful consideration of both risk of recurrence and endocrine responsiveness as a means to better balance the benefits and toxicities from systemic adjuvant therapies. We independently developed two complementary biomarkers, the HOXB13/IL17BR (H/I) index and the molecular Grade Index (MGI) that predict endocrine responsiveness and risk of recurrence, respectively, in early stage ER+ breast cancer. In combination, MGI and H/I define three risk groups: Low risk (low MGI), intermediate risk (low H/I, high MGI) and high risk (high H/I, high MGI). We conducted a study to compare risk stratification generated by MGI and H/I with that generated by the OncotyopeDx assay, the most widely used biomarker in early stage breast cancer. RNA was extracted from formalin-fixed paraffin-embedded sections from all obtainable samples of patients from five institutions in which an OncotypeDX Recurrence Score (RS) was reported. Real-time RT-PCR assays for MGI and H/I were completed and binary results of low and high for both were determined using pre-defined cutpoints. Risk stratification via MGI + H/I for each sample was compared to previously reported OncotypeDX RS's (low, intermediate or high).

Within this cohort of 166 patients, 48%, 45% and 7% had a low, intermediate and high RS, respectively, whereas 69%, 18% and 13% had a low, intermediate and high risk of recurrence, respectively, as assessed by the combined H/I and MGI biomarker. Overall, risk stratifications by RS versus MGI + H/I were significantly correlated ($p < 0.001$) with 81% of patients with low RS also having low risk (low MGI). Risk stratification by H/I + MGI reduced intermediate risk via RS by 2.5-fold (45% versus 18%). MGI + H/I produce a significantly smaller group of patients with intermediate risk by re-stratifying a large number of patients with intermediate RS into low and high risk groups. Risk stratification by H/I + MGI in ER+ patients is by measurement of two discrete parameters: endocrine responsiveness (H/I) and proliferative status (MGI). This may allow for more informed treatment decisions by the treating oncologist. Prospective comparative analysis to assess the clinical significance of these two biomarkers is currently underway.

References: 1. Ma X et al. J Clin. Oncol. 24: 4611, 2006. 2. Wang Z et al. Clin. Cancer Res. 13: 6327, 2007, and Ma X et a.l Clin. Cancer Res. 14: 2601, 2008.

Keywords: HOXB13/IL17BR, breast cancer, OncotypeDX

318 Rapid and Multiplex Molecular Diagnosis and Therapeutic Monitoring of Cancer Based on Real-Time Magnetic Nanotag Sensing

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Reproducible and multiplex assays are greatly desired by cancer biologists as well as clinical oncologists to rapidly follow numerous proteins and nucleic acids in clinical samples in an automated and high throughput fashion. By essentially applying patient serum or tissue samples to the magneto-nano sensor chip developed in our group, one can readily ascertain the presence or absence of a large number of tumor markers, such as those involved in HER-kinase axis pathway. This will allow physicians to determine the efficacy of chemotherapy in real time. The ease of use and low cost inherent in the magneto-nano detection platform is also ideal for early detection of cancer with an appropriate biomarker panel.

We have successfully applied magneto-nano biochips based on giant magnetoresistance (GMR) spin valve sensor arrays and magnetic nanoparticle labels (nanotags) to the detection of biological events in the form of both protein and DNA assays with great speed, sensitivity, selectivity, and economy. The technology is highly scalable to deep multiplex detection of biomarkers in a complex disease, and amenable to integration of microfluidics and CMOS electronics for portable applications. Our results suggest that magneto-nano biochip holds great promises in biomedicine, e.g., for multiplex molecular diagnostics of cancer, infectious diseases, and other diseases. In particular, we have prototyped biochips with an 8 x 8 array of 64 giant magnetoresistance (GMR) spin valve sensors. Each sensor is about 110 um by 110 um in area and covered with a unique DNA or protein feature which can be spotted with a robotic inkjet or other types of pins. The total area of the chip is about 10 mm by 12 mm, while the active 8 x 8 sensor array occupies an area of only 3 mm x 3 mm. The chip is also compatible with our microfluidic sample delivery/washing channels and electronics. The fabricated chips are intrinsically multiplex by virtue of having 64 capture probe spots, enabling multiplex detection of up to 64 biomarkers in one test. Moreover, the sensors under an ultrathin passivation layer have proven to be chemically stable in aqueous solutions or serum samples. These chips are ideal for measuring multiple protein levels in a volume of only 10-50 uL of serum sample, from a human patient with minimal invasiveness in an emergency room or point of care. We have also successfully shown our magneto-nano chip can be used for 4-plex Human Papillomavirus (HPV) genotyping, and multiplex protein assays with a model cancer biomarker panel comprising of TNF-α, VEGF, IL-10 and IL-1α.

This work is supported by National Cancer Institute, National Institute of Allergy and Infectious Diseases, and National Science Foundation.

Keywords: molecular diagnosis, protein, HER

319 DDX3 Associates With DR5 and Negatively Regulates Apoptosis Signal Transduction of the Death Domain

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Therapeutic agents that target death receptors on tumor cells, including the native ligand TRAIL, and agonistic monoclonal antibodies, have been in the clinical development phase. While these agents are shown to benefit some cancer patients, the resistance of tumor cells to TRAIL-mediated apoptosis has been a major concern. Therefore, understanding of the molecular mechanisms by which tumor cells develop apoptosis resistance to death receptors is important in the development of an effective anti-death receptor strategy for cancer therapy. We have previously reported that tumor cells can develop an inducible resistance to DR5-mediated apoptosis via an as yet unknown mechanism. We have identified DDX3, a member of the DEAD Box protein family, as a novel death receptor-associated adapter protein, which associates with a non-death domain region of the cytoplasmic tail of DR5 and recruits cIAP1 via its Caspase Recruiting Domain (CARD), thereby forming an inhibitory complex of DR5/DDX3/cIAP1 to antagonize the death domain function. A mutant form of DR5 lacking the DDX3 binding domain was more pro-apoptotic than the wild-type form. Knockdown of DDDX3 significantly enhanced DR5-mediated apoptosis. A dominant mutant form of DDX3 lacking the CARD reversed apoptosis resistance in tumor cells. The levels of cIAP1 and other apoptosis inhibitory proteins in the DR5/DDX3 complex were correlated with the susceptibility/resistance of tumor cells to DR5-mediated apoptosis. Thus, the DR5/DDX3/IAP complex might serve as a drug target for specific enhancement of DR5-mediated apoptosis, and might be a critical biomarker to predict tumor cell response to DR5-mediated apoptosis.

Keywords: DDX3, DR5, apoptosis

320 Molecular and Functional Imaging of Cancer

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The twenty-first century has witnessed an explosion of molecular biology techniques, amazing advances in imaging, and the design of unique imaging probes. Despite the tremendous strides made in these areas of science, the cure for cancer remains beyond our grasp. Cancer is a complex disease and the apparent impenetrability of the disease is largely due to the multiple, often redundant pathways, which appear to evolve through the genetic instability of cancer cells. The ability to identify and image key common pathways specific to cancer cells, and the ability to image the effectiveness and outcome of strategies designed against these targets is critically important in the treatment of this disease. The vision of our JHU ICMIC Program is to combine state-of-the-art imaging capabilities with powerful molecular biology techniques to define strategies with 'intent to cure'. The JHU ICMIC and JHU SAIRP programs have laid a strong foundation for the establishment of a world class *in vivo* cellular molecular imaging program at Johns Hopkins. Our JHU ICMIC structure consists of four interactive and closely related research components focused on hypoxia, HIF-1, and exploiting the hypoxia response element to target cancer cells through choline kinase inhibition. The research components utilize MR, PET and optical imaging technology to understand cancer vascularization, invasion and metastasis, to achieve effective cancer therapy.

The developmental projects are highly relevant to the goals of the ICMIC and interact with the research components. Five resources devoted to administration, molecular biology, imaging, probes, and translational application provide the infrastructure to support the research activities of the ICMIC. A career developmental program is training the future leaders of molecular imaging in cancer. An advisory board consisting of leading scientists at Hopkins, and at several institutions in the US and abroad, provide critical evaluation of the progress made. Strong institutional support and advocacy ensure the fulfillment of its vision.

Keywords: MR imaging, optical imaging, cancer

321 Molecular Targeting of HER2 for Diagnosis and Therapy of Breast Cancer

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Expression of HER2 receptors in breast cancer is correlated with poor prognosis and may be different in distant metastases as compared to the primary tumor. We are developing methods to assess global expression of HER2 *in vivo* and to target HER2 for delivery of therapeutic agents. As the targeting agent we use an Affibody molecule (http://www.affibody.com). These very stable and highly soluble proteins are relatively small (8.3 kDa) and bind to HER2 receptors with high affinity (22 pM). For imaging with PET, SPECT, or optical methods, an appropriate imaging beacon can be attached to a unique C-terminal cysteine residue of Affibody. We used PET imaging with ^{18}F-Z_{HER2}-Affibody to monitor the down-regulation of HER2 following four doses (50 mg/kg) of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin, 17-DMAG, an inhibitor of Hsp90 known to decrease HER2 expression. Animals were scanned before and after treatment. The results were compared with *ex-vivo* analysis of receptor expression. For optical imaging, we used AlexaFluor dyes conjugated with affibody molecules containing an albumin binding domain that extended their circulation time. For therapy, Affibody molecules are conjugated with thermo-sensitive liposomes that can be labeled with beacons for *in vivo* imaging and loaded with therapeutic agents. Alternatively, Affibody molecules are conjugated with gold nanoparticles that could carry relative large amounts of therapeutic agents and, activated with neutrons, would emit gamma radiation allowing *in vivo* monitoring of their distribution by SPECT. In addition, we have recently developed a recombinant DNA construct combining HER2-specific Affibody molecules with Pseudomonas Endotoxin (PE).

Our results showed that Affibody molecules do not affect the targeted cells and that their binding does not interfere with either the binding or the effectiveness of trastuzumab. ^{18}F-Z_{HER2}-Affibody was eliminated quickly from blood and normal tissues, providing high tumor/blood and tumor/muscle ratios by 1h post injection. The signal obtained from PET and optical imaging correlated well with the number of receptors expressed in the studied tumors as assessed by western blot, ELISA, and IHC. Following 17-DMAG treatment, the level of HER2 expression, estimated by PET imaging, in BT474 and Mcf-7/clone18 tumors decreased 70% and 30%. This change was confirmed by the biodistribution studies, ELISA and western blot. The "Affitoxin" molecule (Affibody-PE hybrid) showed dose-dependent HER2-specific toxicity *in vitro*. Preliminary biodistribution studies using AlexaFluor-labeled Affitoxin showed preferential accumulation of the conjugate in the tumor and in the kidney. The efficacy and toxicity of Affitoxins are currently studied.

This strategy, involving assessment of target presence and distribution in an individual patient followed by optimized, target-specific drug delivery, may significantly improve efficacy of breast cancer treatment while reducing side effects.

Keywords: HER2, molecular imaging, targeted therapy

322 Development of Traceable Syngeneic Mouse Tumor Models With Bifunctional Cell-labeling Lentiviral Vectors

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Cell labeling with bioimaging markers allows one to monitor and track tumors *in vivo*, greatly facilitating the study of mouse cancer models. However, for animal studies the duration of marker gene expression has been highly variable. Lentiviral vectors (LVs) can efficiently transduce reporter genes into a broad spectrum of cell types, achieving stable expression without drug selection. To optimize such vectors for animal study, we have developed a series of pSico-based LVs with different promoters for driving reporter genes. Long-term culture and tumor colony formation of LV-labeled mouse melanoma cells showed that reporter sustainability and consistency was promoter- and cell type-specific. Promoters derived from mammalian house-keeping genes, especially the RNA polymerase II (Pol2) and ferritin (FerH) genes, provided more consistent reporter expression than other types of promoters.

For studies of tumor mouse models, cell labeling vectors were generated in which Pol2 and FerH were used to drive expression of a luciferase-GFP fusion gene (Luc/GFP), namely Pol2- and FerH-Luc/GFP. B16BL6 mouse melanoma labeled with Pol2- or FerH-Luc/GFP could be monitored *in vivo* by bioluminescence (BL) imaging, and a sustained signal was observed for several passages in syngeneic C57BL/6 mice. Moreover, GFP-positive cells could be readily isolated from labeled tumors by fluorescence-activated cell sorter (FACS). The BL signal tracking and GFP-positive ratio in each passage revealed that Pol2-Luc/GFP labeling was more consistent than the FerH-Luc/GFP in this model. In a syngeneic FVB/N mouse model of rhabdomyosarcoma, the Pol2-Luc/GFP labeling was still sustainable but the FerH-Luc/GFP labeling intensity diminished over several *in vivo* passages.

In conclusion, we have characterized an efficient cell-labeling LV, Pol-2-Luc/GFP, which demonstrates promise for use in developing traceable tumor models in conjunction with syngeneic immunocompetent mice, allowing both long-term BL monitoring and FACS-based isolation of tumor cells within animal tissues.

Keywords: lentiviral vectors, mouse tumor models, bioimaging

323 Multifunctional and Long-Circulating Nanoparticles for *In Vivo* Cancer Imaging and Therapy

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The development of biocompatible nanoparticles for *in vivo* molecular imaging and targeted therapy is an area of considerable interest in translational cancer research. The basic rationale is that nanometer-sized particles have functional and structural properties that are not available from either discrete molecules or bulk materials. When conjugated with biomolecular targeting ligands such as monoclonal antibodies, peptides or small molecules, these nanoparticles can be used to target malignant tumors with exquisite specificity and affinity. In the "mesoscopic" size range of 10-100 nm diameter, nanoparticles also have large surface areas for conjugating to multiple diagnostic (e.g., optical, radioisotopic, or magnetic) and therapeutic (e.g., anticancer) agents. Recent advances have led to the development of biodegradable nanostructures for cancer drug delivery, iron oxide nanocrystals for cancer imaging, quantum dots for multiplexed molecular diagnosis, and nanoscale carriers for siRNA delivery.

Here we report the development of targeted and biocompatible nanoparticles for cancer imaging and therapy based on self-assembled and hyperbranched polyglycerols. We developed hyperbranched polyglycerols (PG) as a new carrier for cancer drugs because of their unique structural and biocompatibility properties: (i) PG with polyether backbones is highly water-soluble and biocompatible; (ii) PG is "nonsticky" and can be used to reduce non-specific absorption of proteins and prolong the blood circulation time of nanoparticles; in contrast, other nanoparticles are often coated with plasma proteins and are quickly cleared out by the reticuloendothelial (RES) system *in vivo*; (iii) every repeating unit of PG has one hydroxyl group, which allows the coupling of drug molecules, imaging agents and bioaffinity targeting ligands via established chemistries under mild conditions; (iv) when conjugated with hydrophobic species, PG is able to self-assemble into nearly neutral nanoparticles with a size range of 50-100 nm, a structural feature that is important in reducing the rapid renal clearance of the conjugates. For anticancer applications, these properties become more important since the accumulation of nanoparticles in tumors requires the presence of nanoparticles circulating in the blood for long periods of time. These multiple unique properties have led to greatly enhanced therapeutic indexes of PG-conjugated paclitaxel (TX) compared to free TX on a number of xenograft tumor models including subcutaneous and orthotopic head and neck tumors.

Our results also show that linking an optical imaging probe, such as a near-infrared dye, enables *in vivo* imaging and biodistribution studies of systemically injected nanoparticles. We will present our latest *in vivo* imaging and therapy data including a direct comparison of therapeutic efficacy with FDA-approved nanoparticle drugs. We will also discuss pathways for translating this new class of multifunctional nanoparticle agents to Phase I clinical trials for metastatic and late stage human cancers. (Supported in part from NCI P50 CA128613).

Keywords: nanoparticles, hyperbranched polyglycerols, PG-conjugated paclitaxel

324 Imaging Cancer Treatment Response: PET Translational Research at the University of Washington

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Imaging scientists and cancer doctors at UW have been developing prognosis and prediction methods by characterizing the biochemistry of cancer. Our methods use Positron Emission Tomography (PET) imaging in a multi-disciplinary UW/FHCRC cancer program that has led the way in understanding how to use this powerful molecular imaging modality in applications to cancer. In addition to 3D imaging capabilities to assess heterogeneity, PET provides noninvasive quantitative measurement of tissue concentrations of biologically relevant radiotracers in any body region. Our focus is on general characteristics of the tumor phenotype and traits that might lead to resistance to treatment interventions. Our approach uses knowledge of cancer biology to target pathways that have broad scientific validity, address important clinical needs such as choosing treatment options or assessing treatment response, and are feasible from an imaging perspective. These capabilities have led to development of imaging agents more specific to cancer biology than glycolysis measured by FDG. We image cellular proliferation with C-11 thymidine and F-18 fluorothymidine and cell membrane synthesis using C-11 acetate and are developing F-18 fluoroannexin V for measuring cellular death. Imaging can also be used to assess the spatial heterogeneity and/or time course of resistance factors such as hypoxia (F-18 misonidazole), multiple drug resistance via P-glycoprotein (C-11 verapamil) or estrogen receptor status (F-18 16-α-fluoroestradiol). Other new imaging agents include labeled chemotherapeutics. The use of PET imaging requires development of analysis algorithms for the time course of uptake of each tracer so that delivery can be distinguished from local utilization. For example uptake of proliferation tracers is often dominated by blood flow and a calculated image is needed to measure binding in the salvage pathway versus non-incorporated tracer.

Currently, the group uses combinations of PET agents to probe cancer biology in different histologic types. Imaging for planning treatment for the individual patient or as a pharmacodynamic endpoint are two ways experimental imaging procedures are being used to optimize experimental treatments. In this setting, imaging procedures are validated as reflecting biochemical processes rather than as predictors of clinical outcome. Much of our research is focused on imaging for identifying and understanding cancer treatment responses. Highlights from our recent studies include:

- New parametric models for imaging tissue proliferation can distinguish proliferation from transport and can be used to distinguish radionecrosis from recurrence of brain tumors.
- Hypoxic tumor volume in glioblastoma and HNSCC correlate with survival. Hypoxia images can be used to define a biologic target volume for an image-guided radiotherapy boost.
- Response to anti-vascular therapy can be observed as a change in hypoxia images.
- Estrogen receptor expression can be heterogeneous within an individual and the dose required to saturate ER in tumors can be higher than in normal uterus.
- Multi-tracer studies are feasible to quantify and distinguish resistance factors in patients with sarcomas.

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Keywords: PET imaging, pharmacodynamic endpoints, treatment resistance

325 Phadmacodynamic Study of Sunitinib Therapy Using FLT PET Imaging

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Background: Sunitinib (SU) is a tyrosine kinase inhibitor (TKI) with activity against vascular endothelial growth factor receptors (VEGFR). During VEGFR TKI withdrawal, increase in pain at sites of known metastasis has been observed, which we hypothesize is due to the proliferative flare. [^{18}F]-fluoro-3'-deoxy-3'-L-fluorothymidine (FLT) PET imaging was used as a marker of cell proliferation. By correlating changes in FLT PET, plasma VEGF levels and peripheral blood mononuclear cell (PBMC) HIF1-α expression, we assessed FLT PET imaging as a marker of response, and changes in blood markers as a mechanism of VEGFR TKI escape.

Methods: Patients with advanced solid malignancies and no prior anti-VEGF exposure were enrolled. All patients had metastatic lesions amendable to PET/CT imaging. SU was given at the standard dose of 50 mg PO daily x 4 weeks, followed by a 2 week break (Schedule A, 6 week cycles) or 50 mg PO daily x 2 weeks, followed by a 1 week break (Schedule B, 3 week cycles). FLT-PET scans were obtained at baseline, week 4 (on SU), and week 6 (off SU) for Schedule A or at baseline, week 2 (on SU), and week 1 (off SU) for Schedule B.

Results: 16 subjects have been enrolled to date, 11 subjects on Schedule A and 5 on Schedule B. 7 patients on Schedule A (4 RCC, 1 SCLC, 1 thymus, 1 prostate) had complete sets of FLT PET scans, which were used in analysis. According to the clinical response, the patients were classified in two groups – partial response/stable disease (PR/SD) or progressive disease (PD). Changes in total standardized uptake value (SUV_{tot}) were calculated together with RECIST measurements. According to the RECIST criteria, there was no significant difference between the groups after one treatment cycle (PR/SD: -5%, PD: -8%). On the other hand, change in SUV_{max} between the two groups was significant (PR/SD: -22%, PD: +4%). Interestingly, both groups exhibited a significant increase in SUV_{tot} during withdrawal, but with a significantly higher increase in the PD group (PR/SD: +33%, PD: +120%).

Conclusions: Change in SUV_{tot} appeared to be associated with the degree of clinical response. Proliferative increase in the target lesions during SU withdrawal was seen in all patients to date, supporting the hypothesis that a VEGFR TKI withdrawal leads to a transient period of rapid tumor regrowth. The results of this study support the hypothesis that a continuous dosing schedule of VEGFR TKI might be preferable. Ongoing work is being performed with alternative schedules of SU, assessing pharmacodynamic change with FLT PET.

Keywords: Sunitinib, FLT PET imaging, proliferative flare

326 Cellular and *in Vivo* Activity of a New PI3K Inhibitor-PX866 for Treatment of Human Glioblastoma

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The phosphatidylinositol-3-kinase (PI-3-kinase)/PTEN/Akt pathway is a prerequisite for a wide spectrum of cancers, either via the acquisition of activating mutations in the PI3K catalytic subunit itself, or via loss of PTEN. In this respect class 1 PI3Ks represents well-validated molecules for targeted drug discovery. We demonstrate, herein, that a newly developed PI3K inhibitor PX866, a wortmannin analog with selectivity for p110 α, β and γ (ProlX Pharmaceuticals), effectively inhibits signaling through the PI3K/Akt cascade in a set of glioma cells inhibiting various PI3K signaling components such as Akt, p70S6K1, TSC-2, and pS6. PX866 produces growth inhibitory activities in glioma cell lines with IC50 ranging from 4-8uM. PX866 did not induce apoptotic cell death where as it did induce autophagy- type-2 programmed cell death in U87 glioma cells in a dose dependent manner. In vivo experiment with U87 SC xenograft demonstrated an 84% growth inhibition after 4 weeks treatment at an oral dose of 2.5mg/kg in a qod schedule. PX-866 increased the median survival of animals with i.c.U87 tumors from 31 to 38 days with inhibition of p-AKT and p-S6 as shown by IHC and reverse phase lysate array. To develop a non-invasive method to assess biological activity of PX866, magnetic resonance spectroscopy (MRS) was performed to determine molecular response to PX-866 in the U87 model and tumor volume was assessed by T2 MRI sequence. Tumor volumes dropped on average from 20±11 mm^3 in control animals (n=10) to 5±4 mm^3 in PX-866 treated animals (n=10, $p<0.002$). To assess the potential of MRS-detectable metabolic changes as noninvasive biomarkers of response to PX-866, we performed localized MRS in control and treated animals. Spectra obtained from normal contra-lateral brain differed from spectra of untreated tumors in choline to NAA (Cho/NAA) ratio. Cho/NAA ratio was 0.8±0.1 in contra-lateral brain and increased to 1.7±0.4 (n=10; $p<0.002$) in the untreated gliomas. PX-886 treatment did not affect the spectra recorded from normal brain. In contrast, spectra from the tumor region indicated that Cho/NAA in PX-866-treated tumors was significantly lower by 30% compared to untreated tumors at 1.2±0.5 (n=10; $p<0.02$). Our findings demonstrate that PX-866-treatment results in MRS-detectable metabolic changes indicating a partial normalization of the metabolic profile of treated tumors. Taken together, these data demonstrate that PI3K inhibitor PX866 has significant activity in signal inhibition, cell cycle arrest, growth inhibition and autophagy in human glioblastoma in vitro and in vivo. In addition MRS can be used to non-invasively monitor the molecular effect of PX-866 in gliomas *in vivo* which further confirm the value of MRS in monitoring early molecular response to this PI3K inhibitor in patients *in vivo* affirming that PI3K/Akt pathway is a highly specific molecular target for molecular therapeutics development for glioblastoma and other cancers with aberrant PTEN/PI3K expression.

Keywords: PI3K inhibitor, glioblastoma, imaging

327 Instant Kit Synthesis of 62Cu Radiopharmaceuticals Employing a Distributable 62Zn/62Cu Microgenerator

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Proportional Technologies (PTI) has developed a $^{62}Zn/^{62}Cu$ microgenerator for clinical use in Positron Emission Tomography (PET) imaging. This system supplies the short-lived positron emitter, ^{62}Cu (9.7 minutes) essentially as frequently as desired over a single day and is delivered on a daily basis in a compact and rugged cylindrical stainless steel housing compatible with safe shipment over the U.S. continent. Three *bis*(thiosemicarbazone) lyophilized kits, including H_2ETS, H_2PTSM and H_2ATSM, have been developed for use in concert with the generator to provide onsite instantaneous synthesis of labeled compounds in a 4 mL sterile, pyrogen free, isotonic solution ready for patient injection. The labeling process takes only seconds and achieves > 95% radiochemical purity and requires no special radiopharmacy equipment and no specialized training for clinical personnel.

Full GMP processing procedures and facilities have been developed for production of the microgenerator at PTI's GMP facility in Houston, TX. So far over 50 microgenerators have been produced at PTI under GMP conditions and shipped via overnight courier for use at clinical sites or for external customer studies. All microgenerators shipped from PTI have demonstrated reliable performance in clinical or clinic-like settings using the automated elution equipment. In addition, all of these generators have exhibited negative sterility and pyrogenicity tests. The formulation and development of the lyophilization procedure of the ligand kits have been completed in a 1.5 year development program and finalization of kit production has been achieved. Recently, 800 – 900 unit batches of all three ligand kits were produced under GMP conditions, have passed strict QC testing required by FDA, and are ready for clinical studies.

Commercial IND's for ^{62}Cu-ETS (75018) and ^{62}Cu-ATSM (76897) were filed and approved to begin studies on May 2006 and February 2007, respectively. PTI-sponsored Phase I studies for ^{62}Cu-ETS (n=10, mean injected dose=16.9) and ^{62}Cu-ATSM (n=16, mean injected dose=11.1) have recently been completed to evaluate the safety and biodistribution of these agents through PET imaging in normal volunteers. These studies confirm the safety and absence of toxicity of the ^{62}Cu-ETS and ^{62}Cu-ATSM injectables. Complete quantitative biodistribution/dosimetry results were obtained for both imaging agents. Results indicate the maximum single injection dose for ^{62}Cu-ETS and ^{62}Cu-ATSM to be 33 and 54 mCi, respectively. ^{62}Cu-ETS shows a much improved response to increased blood flow levels and has much lower liver uptake in comparison to the similar perfusion agent, ^{62}Cu-PTSM. Extensive kinetic measurements show that ^{62}Cu-ATSM has the anticipated wash in/wash out behavior in normal tissues that distinguishes it from the perfusion agents. Trapping occurs selectively in the more reducing environment of hypoxic cells.

A Phase II study for ^{62}Cu-ETS was FDA approved on February 2008 and will begin shortly. In this study, preliminary information will be obtained on the efficacy for quantification of regional renal perfusion in two target clinical populations with a full range of renal disease, utilizing the gold standard PET perfusion agent, ^{15}O-water, as a comparator. The safety of ^{62}Cu-ETS will be further assessed in the two patient populations as well as normal subjects. The Phase I final report for ^{62}Cu-ATSM will be filed shortly. Serial measurement of flow and hypoxia using ^{62}Cu-PTSM for perfusion followed by ^{62}Cu-ATSM for hypoxia is a highly promising method made possible by the short half life of Cu-62 of 9.7 min. This methodology provides a widely distributable practical method of measuring two of the most fundamental and important of tumor characteristics in a single PET imaging session. At Duke University, in a PTI-sponsored clinical RDRC study, patients with lung and head and neck cancers are being studied with ^{62}Cu-PTSM/^{62}Cu-ATSM serial imaging in addition to standard of care ^{18}F-FDG. Resulting global and regional tumor hypoxia measures are compared with endogenous markers (carbonic anhydrase IX) and serum markers of hypoxia (VEGF, osteopontin, and PAI-1). To date, five patients have been successfully imaged and analysis of this data is underway.

Keywords: hypoxia, perfusion, Cu-62, Cu-ATSM, Cu-PTSM, Cu-ETS

328 Molecular Imaging of Therapeutic Response to EGF Receptor Blockade in Colorectal Cancer

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Introduction: Non-invasive molecular imaging is capable of visualizing and quantifying cellular and physiological processes *in vivo.* We have evaluated non-invasive molecular imaging methods as correlative biomarkers of therapeutic efficacy of cetuximab in human colorectal cancer (CRC) cell line xenografts grown in athymic nude mice. The correlation between molecular imaging and immunohistochemical analysis to quantify epidermal growth factor (EGF) binding, apoptosis and proliferation was evaluated in treated and untreated tumor-bearing cohorts.

Experimental Design: Optical imaging probes targeting EGF receptor (EGFR) expression (NIR800-EGF) and apoptosis (NIR700-Annexin-V) were synthesized and evaluated *in vitro* and *in vivo.* Proliferation was assessed by [^{18}F]-FLT PET. Assessment of inhibition of EGFR signaling by cetuximab was accomplished by concomitant imaging of NIR800-EGF, NIR700-Annexin-V, and [^{18}F]-FLT in cetuximab-sensitive (DiFi) and insensitive (HCT-116) human CRC cell line xenografts. Imaging results were validated by measurement of tumor size and immunohistochemical (IHC) analysis of total and phosphorylated EGFR, caspase 3 and Ki67 immediately following *in vivo* imaging.

Results: NIR800-EGF accumulation in tumors reflected relative EGFR expression and EGFR occupancy by cetuximab. NIR700-Annexin-V accumulation correlated with cetuximab-induced apoptosis as assessed by immunohistochemical staining of caspase 3. No significant difference in tumor proliferation was noted between treated and untreated animals by [^{18}F]-FLT PET or Ki67 IHC.

Conclusions: Molecular imaging can accurately assess EGF binding, proliferation and apoptosis in human CRC xenografts. These imaging approaches may prove useful for serial, non-invasive monitoring of the biological effects of EGFR inhibition in preclinical studies. It is anticipated that these assays can be used for preclinical modeling of drug combinations and may eventually be adapted for clinical use.

Keywords: EGF receptor, colorectal cancer, cetuximab

329 Effects of Biomechanical Properties of Bone on the Behavior of Breast Cancer Cells Resident in Bone

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Impaired quality of residual bone, as well as decreased bone amount, is common in patients with advanced breast cancer, and leads to skeletal complications that impair quality of life, such as bone pain and pathologic fracture. This is of increasing importance as patients live longer with metastatic disease, and is compounded by current therapies such as aromatase inhibitors. Our hypothesis is that whereas bone loss at the metastatic site is determined by osteoclast activity induced by the breast cancer cells, the quality of the residual bone at the tumor-bone interface is determined by osteoblast differentiation, which is frequently impaired in metastatic breast cancer. Furthermore, we propose that this is a consequence of ambient TGFβ concentrations which are enriched in the microenvironment of metastatic breast cancer cells in bone, and can be decreased by anti-TGFβ therapy, which we hypothesize will enhance osteoblast differentiation as well as improving bone quality. We are examining the elastic modulus of bone at the site of lytic metastases, at the tumor-bone interface, and at sites far removed from tumor in preclinical models. Modulus is being assessed by nanoindentation. We are manufacturing artificial substrates that mimic the biomechanical properties and determining the effects of these substrates on expression profiles in metastatic breast cancer cells.

We are investigating the specific role of TGFβ in osteoblast differentiation, bone structure and quality both in patients and preclinical models of breast cancer metastasis. Preclinical models will provide important information for the design of clinical studies. We are examining the effects of impaired TGFβ signaling in osteoblasts by the use of mice with conditional knockout of the TGFβ receptor kinase, and assessing bone quality as well as bone structure by state-of-the-art techniques including Raman spectroscopy, Atomic Force microscopy and μCT. We are also examining the effects of anti-TGFβ therapy using anti-TGFβ antibodies on bone quality, in parallel with its effects on tumor burden, osteoblast differentiation and bone structure in mice bearing human breast cancer cells, to guide the design of clinical studies, and provide information on the spectrum of benefits from anti-TGFβ therapy. We plan ultimately to evaluate the effects of anti-TGFβ antibodies on bone in a phase I study in patients with metastatic breast cancer. These studies focus on an important complication of breast cancer that markedly influences the quality of life in patients with advanced disease, and should have important therapeutic implications.

Keywords: bone metastasis, TGFβ, bone quality, breast cancer

330 Advances in the Science and Education of Molecular Imaging at Washington University

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Ground-breaking molecular imaging studies have visualized gene expression, biochemical reactions, signal transduction and regulatory pathways in living cells and whole organisms *in vivo*. The Washington University Molecular Imaging Center directs major efforts into fundamental, translational and clinical research under the theme of imaging signal transduction and therapeutics in cancer using novel techniques and probes in combination with genetically-encoded reporters to pinpoint molecular events within target cells. In addition to the exploration of cutting edge translational science, we have begun innovative programs in graduate and undergraduate education in molecular imaging.

Our Molecular Imaging Center activities have spurred several collaborative, interdisciplinary research projects that have formed the foundation for exciting new directions in molecular imaging. This has lead to a major theme of studying regulated signal transduction and protein-protein interactions in living organisms. The principal research projects address imaging of Notch signaling in cancer, investigating cell cycle and checkpoint controls as targets for anticancer agents *in vivo*, investigating viral transcriptional regulation during viral-induced tumorogenicity in doubly transgenic reporter mice, and include a clinical PET study to monitor trafficking of transduced T cells during a clinical suicide gene therapy trial. The program also includes three molecular imaging scientific resources (a molecular reporter core, a chemistry core and a high throughput screening robotics core, founded on molecular imaging platforms for readout of a wide range of bio-assays and libraries) as well as a pilot project program and a multidisciplinary training program for new investigators. As part of the training component, we have developed three new imaging courses for graduate and undergraduate students in Biology, Chemistry, Physics and Engineering, including: Principles and Applications of Biological Imaging (Biology 5146); Contrast Agents for Biological Imaging (Biology/Chemistry 5147); and Biological Imaging Technology (Electrical and Systems Engineering 589).

The ultimate objective of our P50 Program is to combine the institutional expertise of Washington University in the basic sciences of molecular oncology, cell biology and signal transduction with our well developed infrastructure in medical imaging for the advancement of novel interactive and collaborative oncologic molecular imaging projects. Development of imaging coursework has contributed greatly to training students involved in the research described here.

Keywords: signal transduction, molecular imaging, imaging sciences education

331 Clinical Translation of Radiolabeled Cancer Imaging and Therapeutic Agents

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The Radiopharmaceutical Sciences Institute at the University of Missouri-Columbia (MU) is a multidisciplinary center focused on the discovery and clinical translation of novel radiolabeled molecular imaging and therapeutic agents for cancer diagnosis and treatment. The current generation of cancer imaging and therapy agents undergoing clinical translation at MU specifically target cell surface molecules and receptors up-regulated on cancer cells, as exemplified by the receptor targeting alpha-melanocyte stimulating hormone (α-MSH) and bombesin peptide analogs.

A metal cyclized DOTA conjugated α-MSH peptide analog [DOTA-Re(Arg^{11})CCMSH], which targets the melanocortin-1 receptor, was developed as a melanoma imaging and therapeutic agent. The Indium-111 labeled peptide showed high melanoma tumor uptake and retention coupled with rapid whole body clearance kinetics in pre-clinical animal studies, highlighting its potential as a melanoma imaging agent. Results from toxicity testing, performed in accordance with FDA eIND guidance, demonstrated that the non-radioactive compound with or without Indium was not toxic at 100x the estimated patient dose. An eIND application for initial melanoma patient imaging trials is underway. Clinical translation of Lead-212 (^{212}Pb) labeled DOTA-Re(Arg^{11})CCMSH for peptide targeted alpha-particle melanoma therapy is also underway in collaboration with AlphaMed Inc. Preclincial therapy studies with ^{212}Pb-DOTA-Re(Arg^{11})CCMSH in melanoma bearing mice showed a dose dependent survival rate, with up to 45% of the animals melanoma free at the conclusion of the study. Toxicity studies on Pb-DOTA-Re(Arg^{11})CCMSH are scheduled for Fall-08.

Another area of active research interest at MU is in developing diagnostic and therapeutic targeting vectors for the BB2 subtype of the bombesin receptor family. These receptors are known to be upregulated on a variety of human tumors including prostate, breast, small cell lung, and pancreatic cancers. Our group has been successful in obtaining 4 patents on this technology and participated in translating the research for both a diagnostic (^{99m}Tc labeled) and therapeutic (^{177}Lu labeled) BB2r targeting vector from pre-clinical rodent evaluation into human clinical evaluation. Continuing work in this area by our group includes evaluation and development PET agonist and antagonist radiotracers based on ^{64}Cu, ^{68}Ga, and ^{18}F. Further work is underway to develop new methods for ^{99m}Tc labeling to facilitate rapid formulation of these drugs on-site or at a central radiopharmacy. Ongoing preclinical therapeutic trials of ^{177}Lu and ^{90}Y BB2r agonists have demonstrated significant tumor control efficacy when used in combination with cell cycle modifying chemotherapeutics. Current studies are aimed at defining the nature and extent of BB2r expression as a function of therapeutic treatment in vivo using quantitative PET and developing rapid means of in vitro BB2r analysis for high-throughput patient biopsy sample evaluation.

Novel radiolabeled molecules, which specifically target radionuclides to cancer cells, allow physicians to detect cancer at early stages, to monitor the effects of treatment so that patients can be assured to be on the most effective therapy and to treat patients with targeted deposition of therapeutic radiation.

Keywords: melanoma, bombesin, radiopharmaceutical

332 Response of Renal Cell Cancer Mouse Model to Antiangiogenic Therapy Correlates With Tumor Perfusion as Measured With Arterial Spin Labeling MRI

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Purpose: To determine whether Arterial Spin Labeling (ASL) MR imaging can detect early changes in the therapeutic response to antiangiogenic therapy, and whether ASL findings early in the course of therapy can be used to predict later therapeutic response in an animal tumor model.

Method and Materials: The protocol was approved by the institutional animal care and use committee (IACUC) prior to study initiation. Caki-1, A498, and 786-0 human renal cell carcinoma (RCC) xenografts were implanted in 39 nude mice. Animals received 80mg/kg sorafenib qd once tumors measured 12mm diameter. ASL imaging (2mm slice thickness) was performed at baseline and day 14, with additional time points performed for 786-0 and A498 mice at 3days to 12weeks. ASL values were analyzed quantitatively (i.e. using mean blood flow values) and qualitatively (ie. comparing changes in the spatial distribution of blood flow) and compared to the histopathologic reference standard for viability and microvascular density (CD34 staining).

Results: Baseline flow was 80.1±23.3 (ml/100mg/min) for A498, 75.1±28.6 for 786-0 and 10.2±9.0 for Caki-1. Treated Caki-1 showed no significant change (14.9±7.6) in blood flow on day 14, whereas blood flow decreased in all treated A498, with lowest values measured 28-42 days on sorafenib (87.7% mean decrease ± 13.2%) followed by increased blood flow thereafter ($p<0.05$) 17-32 days before documented tumor growth. 786-0 showed decreased mean blood flow on day 3 (20.3±8.7, 73.0% decrease), with no significant changes in mean blood flow until day 22. Although mean blood flow did not change, regions with signal > 2ml/100mg/min seen as early as day 5 correlated to viable tumor.

Conclusion: ASL imaging provides clinically relevant information regarding tumor viability in RCC lines that respond to sorafenib. ASL may also be useful as a predictive biomarker as it documents restoration of perfusion earlier than growth changes in A498. Additionally, low baseline perfusion might predict for lack of responsiveness to antiangiogenic therapy.

Keywords: tumor perfusion imaging, antiangiogenenic therapy

333 Early FDG-PET/CT Predicts Patient Outcome to Preoperative Irinotecan and Cisplatin: Results of a Phase II Therapeutic Study for Locally Advanced Gastric Cancer

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Introduction: Despite the success of preoperative chemotherapy for localized gastric cancer, the majority of patients with locally advanced disease still die of disease recurrence following resection. Identification of chemotherapy resistant disease early in the treatment plan may offer an opportunity for salvage therapy to improve survival. In a prospective phase II study of preoperative irinotecan and cisplatin (NCI 5917), we evaluated the utility of an early change in FDG-PET/CT in predicting response to therapy. In addition, we examined p53 and down stream targets of p53 for correlation to treatment response and survival by immunohistochemistry and microarray technology.

Methods: 42 pts with locally advanced GC(preoperative stage T2N+M0 or T3-4NanyM0) were treated with CPT 65 mg/m2 and CIS 30mg/m2 on day(d)1 and d8, every 21 days for 4 cycles. FDG-PET/CT scans were performed at baseline, and in FDG avid patients, again on d15 and d35. Tissue was obtained prior to therapy by endoscopic biopsy and again at the time of resection for correlative study evaluation.

Results: Pt characteristics are as follows: median age 59(35-77), KPS 90%(70-100%), 27 male, gastric:GEJ 31:11. Median follow up is 23.3 months, with median DFS 23.8 months(95%CI 14-infinity) and median OS 39.1 months(95%CI 31-39months). Pathologic response correlates significantly with DFS(p=0.005) and with OS(p=0.01). Amongst 31 FDG avid pts, a decrease in SUV from baseline to d35 significantly predicts pathologic response(p=0.007) and DFS(p=0.01), whereas the change at d15 does not. 45% decrease in SUV at d35 best distinguishes good from poor pathologic response. With this cutoff, median DFS has not been reached(eg.>23.3 months) for patients with good PET response, and is 14.4 months(95%CI 8.3-infinity) for poor PET responders, p=0.03. Tumors wild type for p53 by IHC were more likely to have a poor pathologic response (p=0.048) and more likely to have early recurrence of disease (p=0.06). Patients without p21 induction had a significantly improved survival over patients with p21 induction (p-0.015).

Conclusions: We confirm that FDG-PET/CT response predicts both pathologic response and DFS following preoperative chemotherapy for locally advanced GC, although at d35. An early PET response provides an opportunity to change therapy in non responding patients, and is currently under investigation. Resistance to irinotecan based chemotherapy may be mediated by p53 and downstream targets. Overcoming p53 mediated drug resistance is currently being tested in a random assignment phase II study in the metastatic setting.

Rationale: The modality being developed is FDG-PET/CT imaging to predict the efficacy of preoperative chemotherapy in locally advanced gastric cancer. This is an image-based assessment tool that is employed when no other imaging technology is available to assess treatment response. This fits the credentialed discovery because it confirms and validates previous studies demonstrating a similar finding, that early FDG-PET scans can predict response to therapy. In conjunction, we are examining the tissue for biomarkers of resistance to therapy. This fits the creation of modality as we are developing clinical markers to predict chemotherapy resistance in gastric cancer.

Keywords: FDG-PET imaging, resistance to chemotherapy, P53

334 Enhancement of Graft-Versus-Tumor (GVT) Effect to Prevent and Treat Relapse After Allogeneic Hematopoietic Cell Transplantation

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Allogeneic hematopoietic cell transplantation (HCT) has efficacy in variety of hematologic malignancies mediated in large part through an immune-mediated graft-versus-tumor (GVT) effect. Despite clinical evidence of a GVT effect , relapse of malignancy is the most common cause of treatment failure and death after allogeneic HCT. The standard therapy for relapsed disease after transplant is the infusion of additional lymphocytes from the donor, referred to as a donor lymphocyte infusion (DLI); however, the efficacy of DLI is quite varied depending upon the histology and bulk of the tumor being treated.. Tumor infiltrating lymphocytes (TIL) having enhanced specificity for tumor antigens have been described in several forms of cancer including malignant melanoma, lymphoma, and breast cancer. One of the major impediments to effective adoptive immunotherapy for cancer is generating sufficient high-affinity, tumor-specific T cells to achieve a clinical response. We have been working extensively to develop a method to generate allogeneic tumor derived lymphocytes (TDL) as a novel form of allogeneic cellular therapy. This method includes CD3, CD28 co-stimulation prior to expansion with IL-2. Based on pre-clinical work from our laboratories, an IND and a clinical protocol for the use of allogeneic TDL for treatment of relapsed B cell malignancies was approved by the FDA in late 2007. This pilot trial will provide new clinical information on the feasibility and safety of administering ex-vivo co-stimulated/expanded TDL in addition to providing data on characteristics of residual tumor after treatment with allogeneic cellular therapy, the immune phenotypic and functional characteristics of TDL, and identification of GVT antigen candidates. Pre-clinical efforts are now directed to the further development of TDL and marrow-derived lymphocytes, to be used for either the treatment and/or prevention of relapse.

Keywords: relapse, tumor-derived lymphocyes, graft-versus tumor

335 Administration of Autologous Cytotoxic T Lymphocytes Targeting EBV Latent Membrane Proteins for the Treatment of Lymphoma

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EBV-associated Hodgkin's Lymphoma (HL) and some non-Hodgkins lymphoma (NHL) show type II latency expressing the subdominant EBV antigens EBNA1, LMP1 and LMP2, which may serve as targets for immunotherapy approaches. In previous studies, we used polyclonal EBV-specific CTL in patients with relapsed EBV +ve HD and saw 2 complete and 1 partial response in 11 patients. Analyses of EBV-CTL lines showed that small populations of T cells reactive against the tumor-associated antigen LMP2 were present in the majority of the infused lines, with some expansion in the peripheral blood following infusion. We therefore hypothesized that CTL specifically targeting LMP antigens might have greater efficacy in these patients. LMP-CTL were generated using Dendritic Cells for initial stimulations then Lymphoblastoid Cell Lines (LCL) both of which had been genetically modified to overexpress either LMP2 alone or LMP1 and LMP2 by transduction with an Ad5f35LMP2 (n=16) or Ad5f35LMP1-I-LMP2 (n=14) vector respectively. All LMP-CTL lines were polyclonal (CD4+ and CD8+). Extensive characterization of these CTL lines revealed specificity for CD4 and CD8 restricted LMP2 epitopes alone (n=15) or both LMP1 and LMP2 epitopes (n=8) (range 1-7 epitopes per CTL line), as determined using overlapping LMP1 and LMP2 peptide pools in ELISPOT assays. Twenty-four patients with EBV+ HL and NHL have been treated on these dose escalation studies (16 with LMP2 CTLs and 8 with LMP1/2 CTLs) No immediate toxicity observed. After CTL infusion, increased LMP-specific T cells were detected in the blood of 15/22 evaluable patients,(range 2 to 70 fold) persisting for up to 3mths. Additionally, two patients had lymph node biopsies 3-6 months post CTL, which showed selective accumulation of LMP2-multimer positive cells in lymph nodes. 12/13 high risk and/or multiply relapsed patients who received LMP-CTL as adjuvant treatment after chemotherapy remain in remission up to 4.5 years after CTL. 11 patients had detectable disease at the time of CTL of whom 2 had progressive disease by 8 weeks and 9 had clinical responses (1 stable disease, 1 very good partial, and 7 complete responses). One of the complete responders was biopsied 7wks after receiving CTL, which were predominantly $CD4^+$ (91.6%). Increased $CD4^+$ T cells were seen compared to pre-CTL biopsy specimens and imaging studies confirmed remission. Immunotherapy with CTL targetting LMP antigens are well tolerated in patients with EBV+ Lymphoma and infused LMP-CTL can accumulate at tumor sites and induce clinical responses.

Keywords: immunotherapy, lymphoma, clinical trial

336 Modulation of Tumor Endothelium for Successful Immunotherapy of Ovarian Cancer

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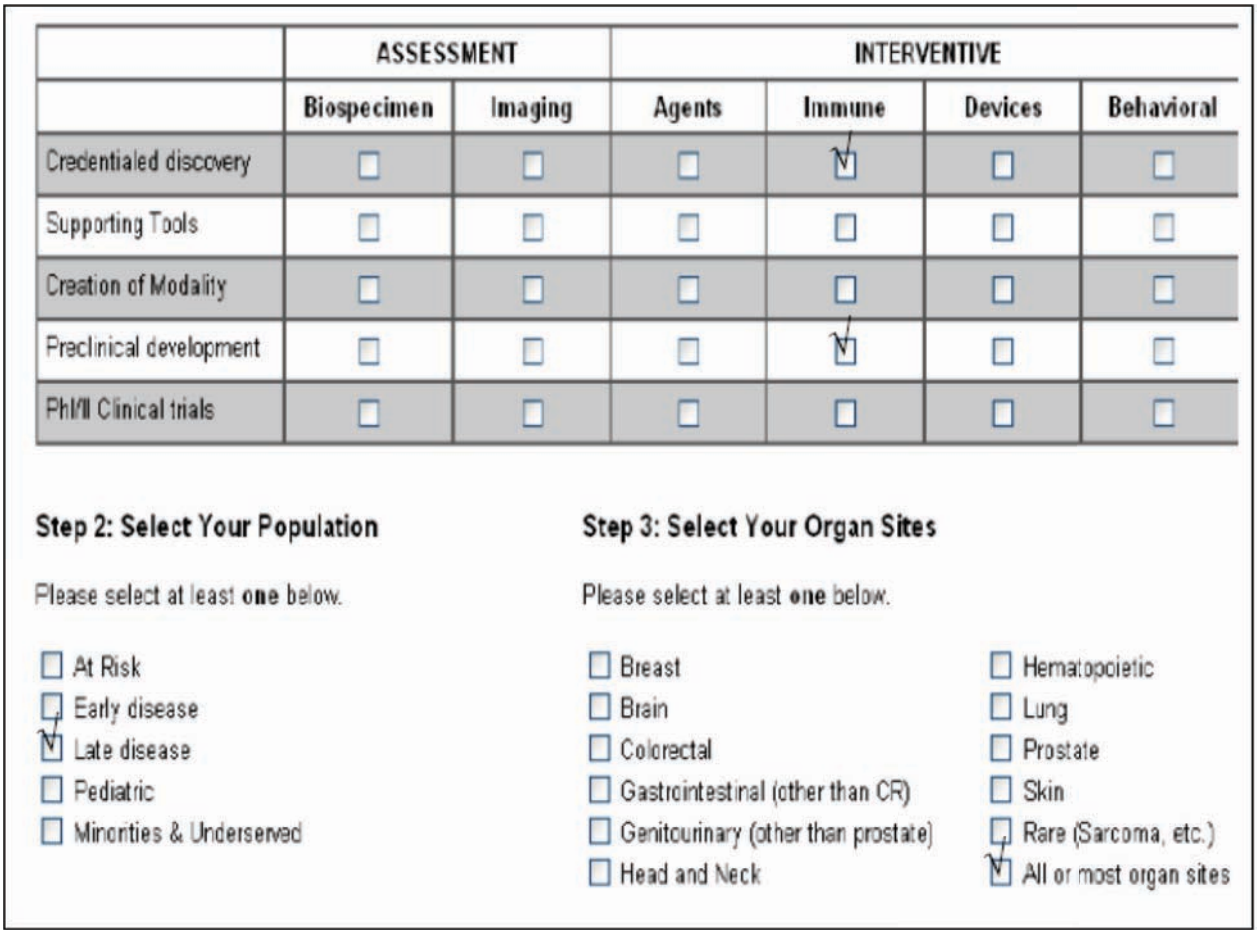
In spite of generating tangible antitumor cellular immune response in peripheral blood, tumor vaccines have proven largely ineffective to date. The mechanisms underlying these failures remain unclear, but tumor microenvironment factors may be implicated. The success of immune therapy partly depends on the ability of effector cells to infiltrate tumors. The mechanisms governing homing of effector cells into tumors remain poorly understood. We hypothesized that tumor endothelium plays a critical role in modulating the homing of T cells. To learn about these mechanisms we studied spontaneous antitumor immune response in ovarian cancer. Transcriptional profiling of microdissected tumor endothelial cells from human ovarian cancers revealed genes associated with absence or presence of tumor-infiltrating lymphocytes (TIL). Overexpression of the endothelin B-receptor (ETBR) was associated with absence of TIL and short survival. We used a specific ETBR inhibitor BQ-788 to evaluate the effects of ETBR blockade in cancer immunotherapy. BQ-788 increased T cell adhesion to human endothelium in vitro, an effect countered by ICAM-1 blockade or NO donors. In the mouse, ETBR neutralization by BQ-788 increased T cell homing to tumors, which required ICAM-1, and enabled tumor response to otherwise ineffective immunotherapy in vivo without changes in systemic antitumor immune response. These findings provide a novel molecular mechanism that can be pharmacologically manipulated to enhance the efficacy of tumor immunotherapy in humans.



	ASSESSMENT		INTERVENTIVE			
	Biospecimen	Imaging	Agents	Immune	Devices	Behavioral
Credentialed discovery	☐	☐	☐	☑	☐	☐
Supporting Tools	☐	☐	☐	☐	☐	☐
Creation of Modality	☐	☐	☐	☐	☐	☐
Preclinical development	☐	☐	☐	☑	☐	☐
Ph/II Clinical trials	☐	☐	☐	☐	☐	☐

Step 2: Select Your Population

Please select at least **one** below.

- ☐ At Risk
- ☐ Early disease
- ☑ Late disease
- ☐ Pediatric
- ☐ Minorities & Underserved

Step 3: Select Your Organ Sites

Please select at least **one** below.

- ☐ Breast
- ☐ Brain
- ☐ Colorectal
- ☐ Gastrointestinal (other than CR)
- ☐ Genitourinary (other than prostate)
- ☐ Head and Neck
- ☐ Hematopoietic
- ☐ Lung
- ☐ Prostate
- ☐ Skin
- ☐ Rare (Sarcoma, etc.)
- ☑ All or most organ sites

Keywords: endothelium, endothelin receptor, vaccine

337 Therapy of CML

Richard Champlin

The University of Texas M. D. Anderson Cancer Center

The goal is to develop novel effective therapy for chronic myeloid leukemia. It is a multidisciplinary program involving molecularly targeted chemotherapy, bio-immunotherapy, allogeneic stem cell transplantation and cellular therapy. There are 8 interactive projects and 6 cores.

Project 1- J. Cortes Project Leader. Chemotherapy for CML. This project has established the efficacy for multiple agents comprising the existing standard of care including interferons, homoherringtonine, imatinib, and novel tyrosine kinase inhibitors.

Project 2- R. Champlin, Project Leader. Stem Cell Transplantation. This project has evaluated the role of allogeneic stem cell transplantation and novel approaches, including the development of intravenous busulfan to optimize pharmacokinetics, leading to FDA approval of this agent. We developed and documented the efficacy of nonmyeloablative stem cell transplantation for CML, and have studied strategies to separate graft-vs.-leukemia effects from GVHD.

Project 3- J. Molldrem. Project Leader. Immunotherapy of CML. This project identified PR1, a peptide derived from proteinase-3, as an immunogeneic leukemia related antigen, and described its role in the immunopathogenesis of CML. Vaccination with PR1 produces clinical remissions in patients with myeloid leukemias related to immunologic anti PR1-T-cell responses

Project 4. S. Kornblau, Project Leader. Suicidal lymphocytes for Separation of GVL and GVHD. This project developed a murine model for the successful use of herpes virus thymidine kinase transduced lymphocytes for inducing antilleukemia responses. These cells can be ablated by treatment with ganciclovir if GVHD develops. A GMP human third-generation vector has been developed and produced for a human clinical trial in myeloid leukemias.

Project 5. Y. Reisner, Project Leader. This project focuses on development of novel strategies to induce tolerance to improve the safety of nonmyeloablative stem cell transplantation. They have demonstrated that cytolytic T-cells raised against an unrelated third party function as veto cells to facilitate engraftment of T-cell depleted transplants and also mediated direct cytotoxicity against a range of hematologic malignancies. The veto effect is augmented by sirolimus and by CD4+CD25+ regulatory T-cells.

Project 6. M. Talpaz, Project Leader. Imatinib resistance. This project demonstrated mechanisms of resistance to imatinib, both bcr-abl dependent and independent.

Project 7. R. Arlinghaus, Project Leader. Inhibition of Normal Hematopoiesis in CML CML is characterized by the progressive replacement of normal hematopoiesis by leukemia cells. These investigators demonstrated that bcr-abl transduced murine cells and human CML cells pathologically produce lipocalin2 (NGAL/24p3) which inhibit normal but not leukemic hematopoiesis. They have also demonstrated an inhibitory role of bcr on the kinase activity of bcr-Abl.

Project 8. M. Andreeff. Project Leader. Mesenchymal Stromal Cells (MSCs) for Delivery of Therapeutic Cytokines. These investigators demonstrated that MSCs localize to the microenvironment of solid tumors and the bone marrow in a model of CML. The MSCs can be transduced to produce interferon-beta which inhibit the growth of the malignancy and delay disease progression. They also demonstrated an important interaction of SDF-1 produced by stromal cells in the microenvironment and CXCR4 expressed by myeloid leukemia cells, which mediates and antiapoptosis effect. Inhibition of this interaction using AMD3100 abrogates this effect and enhances the cytotoxicity of chemotherapy.

Keywords: chronic myeloid leukemia, tyrosine kinase inhibitor, stem cell transplantation

338 Engineering Optimal Hematopoietic Stem Cell Grafts

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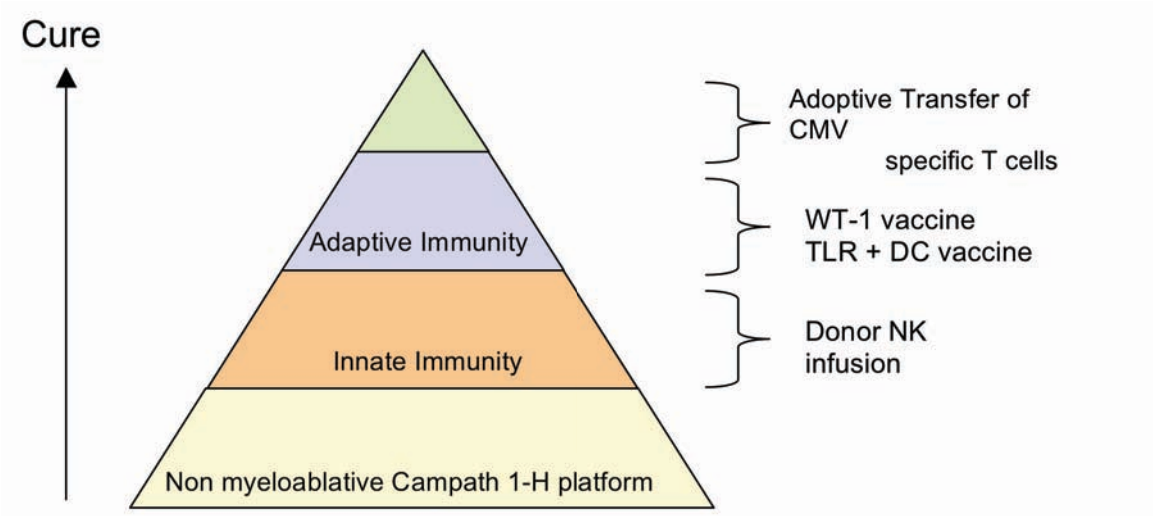
Duke University

The theme of this research effort focuses on improving the therapeutic results and decreasing the toxicity of the preparatory regimens through a collaborative and integrated approach involving the investigators of this program. The central focus is graft engineering, the ability to clearly define the components of a hematopoietic graft so that distinct cellular additions or deletions from a stable platform can be built. As a result of our prior work, we have identified specific areas of investigation to target in an attempt to improve the treatment results. We have established a preparatory regimen of Campath/Fludarabine with Melphalan (lymphoid diseases) or Busulfan (myeloid diseases) upon which we can add or delete different cellular fractions or functions to enhance this base. Our data with the Campath centered non myeloablative regimen allows us to attain a high level of engraftment, with low early treatment related mortality, without excessive graft-versus-host disease (GVHD) in our haploidentical recipients. As expected, with the extensive T cell depletion, we do observe significant infectious complications and relapse. We are now poised to begin several cellular manipulations to improve upon these initial results. These next steps are presented sequentially but there is no a priori reason that they would have to be incorporated into an optimal graft in this fashion. Our first step will be to manipulate the innate immunity. This aim will be accomplished by studying the addition of killer inhibitory receptor (KIR) mismatched donor derived natural killer (NK) cells (Project I) and the importance of toll like receptors (TLRs [Project III]) in an optimal vaccine strategy. In addition to the studies of innate immunity, we will also investigate the adaptive immune system with projects on post transplant immunization against the WT-1 antigen (Project I) and the use of donor derived memory cells to enhance immune recovery without GHVD (Project II). Our plans of graft engineering are graphically demonstrated in the figure below.



It is important to stress that all the lessons learned from these studies in our haploidentical recipients are broadly applicable to more conventional allogeneic hematopoietic cell transplantation. Moreover, these approaches, while represented in a linear fashion above, are in fact highly interrelated and supportive of one another. The synergies that are present in this application will enhance the scientific endeavors of each of the projects. Ultimately, it may be that several of the components mentioned above will need to be combined for the most optimal outcome for our patients. This synergistic process has been one of the strengths of this program and has led to the refinement of our graft engineering strategy.

Keywords: hematopoietic stem cell transplantation, NK cells, immunity

339 Novel Antigens Differentially Expressed in Pediatric Leukemias and Solid Tumors Are Promising Targets for Antibody and Cell-Based Immunotherapies

N.K. Cheung, Kim Kramer, Shakeel Modak, Brian Kushner, Hong Xu, Geunther Koehne, Ekaterina Doubrovina, Steven Larson, **Richard J. O'Reilly**

Memorial Sloan-Kettering Cancer Center

Our Program Project is a multidisciplinary program which has, as its overall objective, the identification, functional analysis and targeted therapeutic modification of gene products uniquely or differentially expressed by malignancies of childhood. From these studies, several proteins have been identified which are differentially expressed in certain tumors, of which two have been selected for evaluation as targets for antibody or cellular immune therapy respectively: WT-1, the Wilms tumor protein, B7H3, a member of the B7 family of immunomodulating molecules and

4Ig-B7H3, the long and principal form of B7H3 in humans, inhibits NK cells and T cells. While B7H3 transcript is ubiquitously expressed in solid tumors and normal human tissues, 4Ig-B7H3 protein is absent in most normal tissues including the central nervous system (CNS). However, it is expressed at high levels on the cell surface of neuroblastomas, certain sarcomas and gliomas. We developed and preclinically tested a murine monoclonal antibody, 8H9, which has high affinity for 4Ig-B7H3. Recently, we have used it to deliver intrathecal radioimmunotherapy (RIT) for primary and metastatic CNS tumors. In a phase I study, a subset of 15 patients with recurrent neuroblastoma metastatic to the CNS were treated following neurosurgery, craniospinal radiation, and chemotherapy with intra-ommaya ^{131}I-8H9. Outpatient 3F8/GMCSF immunotherapy, 13-cis-retinoic acid and oral temozolomide were used to prevent systemic metastases. Thirteen of 15 RIT-salvage patients remain free of CNS neuroblastoma 6-58 months after CNS event, with 11 in complete remission. One patient died of infection at 22 months with no evidence of disease at autopsy, and one of lung and bone marrow metastases at 15 months. The RIT-salvage regimen was also well tolerated. In contrast, 31 of 33 patients receiving treatment without RIT have died. The median time to death was 5.8 months for those with systemic and CNS disease and 11.5 months for those with isolated CNS relapses. The RIT-salvage regimen for CNS metastases was well tolerated by young patients, despite their prior history of intensive cytotoxic therapies. RIT targeting an immune inhibitory molecule differentially expressed by solid tumors in the CNS thus appears safe and effective.

WT-1 is highly expressed in the blasts of over 80% of ALLs and AMLs in children. We have sensitized T cells from normal donors with a pool of overlapping 15-mer peptides spanning the sequence of WT-1, loaded on either autologous DCs or EBV BLCL. T-cells so generated have been characterized as to their WT-1 peptide specificity and HLA restriction. In normal donors, T-cells are responsive to 1-2 epitopes presented by 1-2 class I and /or II HLA alleles. T-cells reactive against epitopes presented class I alleles were CD8+ and lysed WT-1+ leukemia blasts coexpressing the restricting allele. Of 5 class II restricted WT-1 specific T-cell lines, 3 were also leukemocidal. In NOD/SCID mice bearing multiple WT-1 leukemic and sarcoma xenografts, these T-cells selectively migrate to, and induce regression of tumors coexpressing WT-1 and their restricting HLA allele. Using the assay of Dyck et al, we also showed that these WT-1 specific T-cells can eliminate clonogenic leukemia cells. Based on these and other studies, a Phase I trial of WT-1 peptide pool sensitized T-cells has been initiated in leukemic patients relapsing post allogenic HSCT. These initial studies suggest that proteins and processed peptides differentially expressed in childhood malignancies may be specifically targeted in vivo by antibody or cell-mediated immunotherapies.

Keywords: intrathecal immunotherapy, 8H9 antibody, WT-1 specific T-cells

340 Chimeric Antigen Receptor (CAR)-Modified Virus Specific T Lymphocytes for Neuroblastoma

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Neuroblastoma is the most common extracranial tumor of childhood. Children with advanced neuroblastoma have a poor outcome despite intensive therapy. Although, clinical remission can be achieved, relapse is common, especially in high risk patients.

Cytotoxic T lymphocytes (CTLs) defend against viral disease and have been employed as targeted agents to treat cancer. When the CTLs' native receptors are directed to persistent viruses, the cells survive and retain long-term effectiveness in humans. By contrast, CTLs directed to nonviral tumor-associated antigens often show limited survival and activity *in vivo*, in part because tumor cells typically lack appropriate costimulatory molecules. We therefore engineered Epstein-Barr virus (EBV)-specific CTLs to express an additional synthetic chimeric antigen receptor directed to GD2, a nonviral tumor-associated antigen expressed by human neuroblastoma cells. We reasoned that these genetically engineered lymphocytes would receive optimal signals following engagement of their *native* receptors, enhancing survival and antitumor activity mediated through their *chimeric* receptors. Here we show in neuroblastoma patients that EBV-specific CTLs expressing a chimeric GD2-specific receptor indeed survive longer than activated T cells expressing the same chimeric receptor but lacking virus specificity. Infusion of these genetically modified cells appeared safe and was associated with tumor regression or necrosis in half of the patients with evaluable disease. Hence, virus specific CTLs can be modified to function as tumor directed effector cells.

These observations support the benefits of activation through the native αβTCR by EBV antigens and co-stimulatory molecules on APC.

Keywords: neuroblastoma, immunotherapy, chimeric antigen receptor

341 On-Chip Diagnostic Immunoassays of Panels of Bioselected Tumor Antigens: Binding to Serum Autoantibodies for the Early Detection of Cancer

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Introduction to Technology: The humoral immune response is a sensitive biosensor of novel proteins expressed by tumor cells and thus amplifies the signal of new protein changes in cancer. Panels of tumor antigens provide a sensitive and specific multi-analyte immunoassay for pre-symptomatic cancer. In lieu of single biomarker molecules, we use a high-throughput cloning method to identify panels of epitopes/antigens that react with autoantibodies to tumor related proteins in the serum of cancer patients. The binding properties of these serum antitumor antibodies to antigen arrays and using advanced bioinformatics tools has led to the identification of a panel of diagnostic antigens. By gene sequence identification of these antigens, we have discovered a number of novel disease-related proteins for diagnostic studies. There are numerous advantages to employing serum antibodies as the analytes especially the ability to adapt these assays to standard clinical platforms.

Results: We determined the human serum IgG binding profiles for both cancer patients and healthy controls to our cloned antigens using a two fluorescent dye approach. Cloned antigens are generated using a T7 based phage display system and then robotically spotted onto nitrocellulose coated glass slides. Detection of human IgG binding is done using an Alexa Fluor 647 (red fluorescent dye) labeled goat anti-human IgG (Molecular Probes). To account for variations in the amount of antigen clones robotically spotted, antigen arrays are treated with a mouse T7 capsid monoclonal antibody that recognizes all clones and then detected with an Alexa Fluor 532 (green fluorescent dye) labeled goat anti-mouse IgG. The dye ratio data generated from this approach were used to determine the normalized IgG binding profile of each subject's serum to our antigen arrays. All data analyses were conducted in R environment utilizing the Limma, Marray, RandomForest, and RWeka (implementation of Weka in R) packages. The red over green channel dye intensity ratios were log transformed, and the data were normalized to the print-tip group median within each array. The data were randomly split into training and test sets.

We selected 91 antigen clones from the training set that gave significantly different signals between cancer and control samples by Mann-Whitney U-Test (false discovery rate adjusted $p<0.05$). Out of the 91 clones, we selected the best combination of six clones with the Random Forest backward elimination algorithm that used out of bag error rate as a criterion. The top six markers were used to build a Bayesian Network model on the training set and then their classification performance was evaluated on the test set. On an independent test set of cancer and control samples which had not been previously used in any part of the development of these classifiers, the model with 91 markers had an accuracy of 85%, and using the 6 marker model, we achieved 80% accuracy.

Conclusions: This method of detection and qualification of potential biomarkers for cancer exploits the immune system as a biosensor. This is a unique technological platform combining microarrays of cloned antigens for cancer detection and novel bioinformatics systems. The concept, in general, employs pattern recognition of multiple markers as a diagnostic tool for the detection of early stage cancer rather than attempting to use a single marker to predict occurrence or disease outcomes.

Keywords: protein microarrays, autoantibodies, early detection

342 Adoptive Cell Therapy for Patients With Metastatic Melanoma: Evaluation of Intensive Myeloablative Chemoradiation Preparative Regimens

Mark E. Dudley, James C. Yang, Richard Sherry, Marybeth S. Hughes, Richard Royal, Udai Kammula, Deborah E. Citrin, Susan F. Leitman, Armen Thomasian, Paul F. Robbins, John Wunderlich, Nicholas P. Restifo, Steven A. Rosenberg

Surgery Branch and Radiation Oncology Branch, National Cancer Institute, and Department of Transfusion Medicine, Clinical Center, National Institutes of Health

Metastatic melanoma is an aggressive disease with only the two treatments currently approved for use by the FDA, interleukin-2 (IL-2) and dacarbazine, which mediate objective response rates of 12-15%. Preliminary clinical studies demonstrated that adoptive cell therapy (ACT) with autologous anti-tumor lymphocytes after lymphodepleting chemotherapy mediated objective responses in 51% of 35 patients.

To evaluate the safety and efficacy of increased intensity myeloablative lymphodepleting regimens, we performed two additional sequential trials of ACT with autologous anti-tumor lymphocytes (TIL) in patients with metastatic melanoma. Increasing intensity of host preparative lymphodepletion consisting of cyclophosphamide and fludarabine with either 200cGy (25 patients) or 1200 cGy (25 patients) total body irradiation (TBI) was administered prior to cell transfer. Objective response rates by RECIST criteria and survival were evaluated. Immunologic correlates of effective treatment were also studied

While non-myeloablative chemotherapy alone showed an objective response rate of 49% (43 total patients treated), when 200cGy or 1200cGy TBI was added the response rates were to 52% and 72% respectively. Ten patients demonstrated complete responses that were ongoing as of April 2008. Responses were seen in all visceral sites including brain. There was one treatment related death in the 93 patients. Host lymphodepletion was associated with significantly increased serum levels of the lymphocyte homeostatic cytokines IL-7 and IL-15. Objective responses were correlated with the telomere length of the transferred cells. Host lymphodepletion followed by autologous TIL transfer and IL-2 results in objective response rates of 50-70% in patients with metastatic melanoma refractory to standard therapies.

Reference: Dudley M et al., J. Clin. Onc. 2008 in press.

Keywords: melanoma, T cell, interleukin-2

343 Allograft Engineering Using Rapamycin-Resistant Donor Th2 Cells

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Using murine models of allogeneic hematopoietic stem cell transplantation (allo-HSCT), we found that ex vivo generated Th2 cells manufactured in high-dose rapamycin (Th2.R cells) improves the balance of beneficial and detrimental allogeneic T cell effects. Specifically, allograft augmentation with Th2.R cells yielded a balanced Th1/Th2 cytokine profile post-HSCT that resulted in graft-versus-tumor (GVT) effects with reduced graft-versus-host disease (GVHD); we also determined that delay in Th2.R cell therapy until day 14 post-HSCT yielded sequential Th1 ➔ Th2 immunity, which further enhanced a GVT effect yet still allowed an amelioration of ongoing GVHD. In experiments using cytokine-deficient donor Th2.R cells, we determined that the Th2.R cell anti-GVHD effect required both IL-4 and IL-10 production. Furthermore, in a murine model of graft rejection, we found that donor Th2.R cells prevented fully MHC-disparate rejection with reduced GVHD; Th2.R cell promotion of alloengraftment required Th2 cell IL-4 production and host T cell STAT6 signaling, and was associated with host T cell tolerization rather than clonal deletion. To clinically translate these findings, we developed a cGMP method for the manufacture of human Th2.R cells: donor $CD4^+$ T cells were purified by positive selection (Miltenyi® columns), co-stimulated with anti-CD3, anti-CD28 coated magnetic beads, and expanded in IL-4, IL-2, and high-dose rapamycin (1 μM) for 12 days; the resultant Th2.R cells secreted increased IL-4, IL-5, IL-10 and IL-13 (Th2 cytokines) and reduced IFN-γ (Th1 cytokine). An investigator-sponsored Investigational New Drug Application was established (sponsor, Dr. Fowler), and a pilot clinical protocol was initiated to evaluate donor Th2.R cell infusion in the setting of HLA-matched sibling allo-HSCT. Patients with refractory hematologic malignancy received reduced-intensity conditioning with fludarabine and cyclophosphamide (total Cy dose, 4800 mg/m^2) and GVHD prophylaxis of cyclosporine and short-course sirolimus to day 14 post-transplant. Infusion of Th2.R cells (2.5 x 10^7 cells/kg recipient body weight) was associated with a dose limiting toxicity in the form of engraftment syndrome (ES), which was observed in 7/9 Th2 cell recipients. We reasoned that this cell product toxicity might be alleviated by reducing the intensity of host conditioning and by delaying Th2.R cell infusion after engraftment has occurred. Indeed, we have seen no case of ES in 37 consecutive patients treated with at least a 75% reduction in Cy conditioning and Th2.R cell infusion at either day 0 or day 14 of transplant. Timing of Th2.R cell infusion differentially influenced alloengraftment: day 0 therapy tended to promote stable mixed chimerism whereas day 14 therapy tended to associate with prompt progression to full donor elements. Acute GVHD has been reduced relative to our historical control experience (<20% incidence of grade II-IV GVHD). In conclusion, donor Th2.R cell infusion shows promise as a new approach to facilitate alloengraftment after minimal host conditioning.

Keywords: Th2 cells, immune therapy, allogeneic hematopoietic stem cell transplantation

344 Tumor-Initiating Melanoma Cells Require New Strategies for Therapy

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Melanoma cells readily adapt to a new environment such as growth in tissue culture or immunosuppressed mice. Success rates of up to 80% for establishing permanent melanoma cell lines in tissue culture are likely due to the remarkable plasticity of the malignant cells, which is also manifest in the poor responses to chemo-, immuno-, bio-, or radiotherapies that have been tested over the last thirty years, leaving the five-year survival rates for advanced metastatic melanoma at around 15%. Using self-renewal, differentiation and tumor formation in NOD/SCID/IL2Rγ2a$^{-/-}$ mice as criteria, we have defined several melanoma sub-populations as tumor-initiating cells, including CD20^{+}, CD133^{+}, p75NGFR^{+}, and ABCG2^{+} cells. Each of these sub-populations also readily proliferates as 'neuro-spheres' (melanospheres) in media designed for human embryonic stem cells. Melanosphere cells are highly tumorigenic in immunodeficient NOD/SCID/IL2Rγ2a$^{-/-}$ mice. Injection of only 200 cells induced tumors in all animals within a 12-week observation period. However, cells not enriched for tumor-initiating cells and growing in conventional media were also tumorigenic at the same cell dose, albeit tumors grew slower and were less vascularized. Cells from either populations injected at only 20/mouse induced tumors in 50% of the animals suggesting that the majority of melanoma cells of any population is tumorigenic challenging a 'classical' concept that tumors consist of a large population of non-tumorigenic cells. We then identified a sub-population, which appears most critical for sustaining tumor induction. Cells from this sub-population are non-proliferative and are highly resistant to chemotherapeutic compounds. Removal of the non-proliferating sub-population lead to an initial increase in growth *in vitro*, followed by subsequent slow-down and death. Similar exhaustion of cell growth was observed *in vivo*. While the tumors grew in their first passage as rapidly as controls, they slowed down in the second passage and were no longer proliferating after the third passage. These results suggest that a non-proliferating population within tumors is most important for self-renewal and long-term tumor maintenance. This population appears responsible for the resistance to current therapies, thus requiring novel strategies to eliminate it for successful treatment.

Keywords: melanoma, therapy, tumorigenicity

345 Treatment of Epstein Barr Virus Positive Nasopharyngeal Carcinoma With Adoptively Transferred Cytotoxic T Lymphocytes

Heslop HE, Louis CU, Straathof K, Gerken C, Lopez T, Bollard CM, Gresik MV, Liu H, Gee A, Brenner MK, Rooney CM, Gottschalk S

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The prognosis of patients with loco-regionally bulky nasopahryngeal cancer, the most common form at presentation, is poor as many patients develop distant metastases. It is therefore desirable to develop novel therapies that could improve disease-free survival in patients with bulky disease and reduce the incidence of long-term treatment related complications in all patients. The strong association of NPC with EBV makes adoptive immunotherapy with EBV-specific cytotoxic T cells (EBV-CTL) an attractive therapeutic option. We have evaluated the safety and efficacy of EBV-specific CTL (EBV-CTL) in two Phase I clinical trials. A total of 32 patients with advanced-stage NPC have received autologous EBV-CTL. Prior to adoptive transfer, 8 patients were in remission, 22 had active disease, and 2 had abnormal imaging studies of unknown significance. Seven of 8 patients in remission prior to CTL infusion remain in remission 6 - 64 months post CTL. For the remaining 24 patients, the best overall response rate was 50% with 6 complete responses (CR/CRu), 2 partial responses, and 4 with stable disease during a median follow-up of 9 months (95% CI 2 - 16 months). Of the 6 with a CR: 4 have been sustained for 2 - 4 years, and 2 relapsed more than 2 years post CTL. While EBV-CTL therapy has yielded promising results, their efficacy is limited because CTL generated by standard methods are dominated by T-cell clones not reactive to the EBV proteins LMP1 and LMP2 expressed in NPC, and they are sensitive to several immune evasion strategies including downregulation of MHC class I expression and antigen processing defects employed by the tumor. We hypothesize that overcoming these limitations will enhance the anti-tumor activity of infused CTLs and improve treatment outcome. Thus, we have modified our CTL production protocol to prepare EBV-LMP1 and LMP2-specific CTL (LMP-CTL) whose safety and anti-tumor activity is being investigated in a Phase I clinical trial which has already accrued 8 patients. To render LMP-CTL resistant to MHC class I processing defects, we plan to express a chimeric antigen receptor (CAR) on LMP-CTL. Our preliminary results have already identified 3 potential non-EBV antigens expressed on the cell surface of NPC that could be targeted with CARs: CD70, HER2, and EGFRvIII, a splice-variant of EGFR. We are currently evaluating in preclinical studies the anti-NPC activity of CAR.LMP-CTL, and if successful will develop a Phase I clinical study to evaluate the safety and efficacy of this approach. Although we are focusing on adoptive immunotherapy for EBV-positive NPC, the information obtained will be applicable to cellular immunotherapies for other tumors with defined tumor antigens.

Keywords: nasopharyngeal cancer, EBV, CTL

346 GVL Without GVHD Across HLA Barriers: Translational Studies: Genetics and Mechanisms of DLI Effects Following Non-myeloablative HCT

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Non-myeloablative hematopoietic cell transplantation (HCT) followed by delayed donor leukocyte infusion (DLI) is a promising immunotherapeutic approach to treat hematologic malignancies including leukemias and lymphomas. Major limitations of this approach, however, include the risks of graft failure, graft-versus-host disease (GVHD), and infection. MGH MHC-defined miniature swine provide a pre-clinical model with responses to HCT that closely resemble those in humans. Similar to what would be expected in the clinic, animals that receive haploidentical HCT following standard myeloablative conditioning developed severe GVHD or hematopoietic failure.

In this study, we investigate novel approaches for HCT that harness the immunomodulatory capacities of the immune system, rather than completely ablate donor and host T cells, to promote better engraftment with fewer complications. We demonstrate successful haploidentical HCT in miniature swine with a minimally myelosuppressive regimen consisting of a very low-dose of total body irradiation (100cGy TBI) in combination with transient recipient T-cell depletion and a short course of cyclosporine A (CyA). Strikingly, GVHD is not observed in engrafted animals despite the fact that an enormous number of donor T cells (>5 x 10^9 T cells/kg) are infused with the initial HCT. Our data demonstrate that regulatory cells play an important role in preventing GVHD and may facilitate engraftment and tolerance in this model. These data suggest that mild HCT protocols that preserve regulatory cell development and function may be more successful in achieving stable engraftment and avoiding GVHD than those that rely on harsh conditioning and complete donor and host T-cell depletion. This is particularly true for large animals and patients since complete donor and host T-cell depletion has proven to be a very difficult and risky approach.

Our data also demonstrate that DLI fails to convert chimeric swine to full (donor) chimerism, a process associated with powerful anti-tumor effects. It is possible that the same immune regulatory cell mechanisms responsible for facilitating engraftment while controlling GVHD may also interfere with DLI-mediated tumor immunotherapy. Preliminary data suggest that leukodepletion of chimeric recipients prior to DLI improves DLI effects. Progress has been made toward the development of a transplantable myeloid leukemia model in miniature swine to enable direct assessment graft-versus-leukemia (GVL) effects of HCT and DLI in this model. Our goal is to understand the immunological mechanisms that permit engraftment without GVHD, but also interfere with DLI effects, so we can manipulate these mechanisms to further improve HCT and DLI outcomes in the clinic.

Keywords: hematopoietic cell transplantation, tumor immunotherapy, miniature swine

347 Semi-Continuous Intralymphatic Infusion of Dendritic Cells as Therapy of Advanced Melanoma (UPCI 03-118)

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Cancer vaccines applied as therapy need to induce high numbers of effector-type tumor-specific CTLs and Th1 cells that can enter tumor tissues and perform anti-tumor functions. Therapeutic vaccines also need to function in the presence of T*regs* and DC-killing $CD8^+$ CTLs present in peripheral tissues of cancer-bearing individuals. In attempt to improve the efficacy of cancer vaccination, we have developed type-1-polarized dendritic cells (αDC1s) with selectively-enhanced ability to activate cancer-specific CTLs and Th1 cells, rather than T*regs*. In order to assure rapid delivery of "non-exhausted" DCs to the lymph nodes and to avoid their inactivation/elimination by peripheral T*regs* and CTLs, we have developed a semi-continuous intralymphatic mode of vaccine delivery, using an implantable lymphatic cannula. This approach allows the efficient and rapid delivery of vaccines to draining lymph nodes without disruption of the nodal structure. It also allows for repeated/semi-continuous delivery of vaccines over prolonged time periods, mimicking the kinetics of the migration and persistence of functional DCs during physiologic immune responses.

We have completed the initial safety evaluation of standard- and αDC1-based intralymphatic vaccines (25,000 DC per injection; a total of 300,000 DCs over 4 days) in patients with stage IIIb-IV melanoma in trial UPCI 03-118 and are now proceeding to the comparative evaluation of "high" doses (250,000 DC per injection). Unexpectedly, in addition to the documentation of feasibility and safety of this prolonged lymphatic cannulation and semi-continuous delivery of DC (12 injections over 4 days), we have already observed evidence of clinical efficacy at this ultra-low dose-level (10-100 fold fewer DCs than routinely-used doses of intradermal or intranodal vaccines). Among the first four patients who completed two courses of vaccination, one partial antitumor response was observed in a patient with stage IIIb disease (near-complete and of >12 months duration), and one stabilization of stage IV disease (lung; over 6 months long).

The current data demonstrate the feasibility of prolonged intralymphatic delivery of biotherapeutic agents in patients with advanced cancer and provide preliminary indication that DC-based cancer vaccines can be clinically effective even at ultra-low doses, up to 100-fold lower than the doses currently considered as necessary.

Keywords: vaccines, melanoma, dendric cells

348 Plasma-Based ELISA Assay to Monitor Melanoma Patients for Recurrence and Response to Systemic Therapy

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Tumor-derived proteins have been identified in the blood of patients with unresected disease in a number of malignancies. These proteins are used to monitor patients for disease recurrence and changes in disease burden with the administration of systemic therapy. Examples include CA 15-3 and CA 27.29 in breast cancer, CEA in breast and colon cancer, PSA in prostate cancer and CA 125 in ovarian cancer. Our goal is to devise similar blood tests for melanoma patients.

Our strategy was to focus on proteins that are highly expressed in melanoma cells derived from patient tumors. NimbleGen whole genome exon arrays were employed to determine gene expression levels in 11 melanoma cell strains derived from resected advanced or metastatic tumors from different patients. Our priority was to study highly expressed genes (hybridization intensity levels >10,000), encoding secreted proteins, plasma membrane proteins and extracellular matrix proteins that are likely to be secreted from the cells, for which ELISA kits are commercially available. This list includes MIF (macrophage migration inhibitory factor), CEACAM (CEA related cell adhesion molecule), IL-8, OPN (osteopontin), MIA (melanoma inhibitory activity) and GDF-15 (growth differentiation factor-15). Levels of these proteins were assessed in plasma from 24 patients with stage I-II melanoma, 10 with stage III (before surgical resection), 66 stage IV patients and 82 healthy donors.

Unpaired t-tests determined that levels of MIF, CEACAM, and OPN were higher in plasma samples from melanoma patients than those of healthy individuals ($P=0.03$, $P=0.01$, $P= 0.0014$, respectively), while levels of IL-8 and GDF-15 were not significantly different ($P=0.4$ and $P=0.08$, respectively) in these two groups. There were no differences in the levels of any of the markers in plasma samples from healthy individuals and stage I-II patients, suggesting that the differences are due to higher levels in stage III and IV patients. Differences between patients with stage I-II disease and stage III-IV disease were seen for CEACAM, IL-8, OPN, MIA and GDF-15 ($P=0.0038$, $P=0.02$, $P=0.002$, $P<0.0001$ and $P=0.028$, respectively). Cut-points were determined for "high" and "low" protein level by the 95^{th} percentile level in normal individuals. Plasma samples of 50% of stage III patients had high levels of at least one marker, whereas 79% of plasma samples from stage IV patients had high levels of at least one marker. Given the clear association between the presence of these markers and disease stage, we assessed whether changes in marker level in the plasma were associated with response or progression on therapy for 12 patients, who had at least three serial measurements of these markers. There was a decrease in plasma protein levels in three out of four of the responders, an increase in five out of six patients whose disease progressed, and no clear differences were seen in two patients with radiographically stable disease.

Abundantly expressed genes in melanoma tumors can be used as an initial tool for selection of putative plasma markers that might be useful for monitoring patients for disease recurrence. Such a plasma test might supplement or compliment radiographic studies in monitoring metastatic disease patients receiving systemic therapy. Additional markers are being studied and prospective validation of these findings is ongoing.

Keywords: CEACAM, MIF, OPN

349 Identification and Validation of Immune Biomarkers as Predictors of Vaccine Response

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The broad objective of our work is to develop new methods for identifying and validating immune biomarkers of response to immunotherapies for the treatment of pancreatic cancers. Vaccine based therapies are currently under all stages of clinical development. Although these therapies have shown some promise in early development, understanding the mechanisms by which these therapies are functioning in patients has been limited due to the lack of immune relevant antigens that can be evaluated at a molecular level. Recent developments in molecular and proteomic technologies have allowed the development of functional approaches for identifying relevant immune targets expressed by cancers. Through the Spore program, we have been developing an allogeneic granulocyte-macrophage colony-stimulating factor (GM-CSF) secreting pancreatic tumor vaccine approach for the treatment of pancreatic cancer. Completed phase I and II studies demonstrated the bioactivity of this vaccine as measured by elevated post-vaccination serum levels of GM-CSF, post-vaccination eosinophilia that is associated with systemic vaccine related rashes and vaccine recall reactions, and post-vaccination delayed type hypersensitivity (DTH) reactions to autologous tumor. These responses are most often observed in patients demonstrating prolonged disease-free survival. Analysis of post-vaccination immune responses identified mesothelin and prostate stem cell antigen (PSCA) as two candidate new targets against which both T cell responses were directed in patients who remain disease-free. More recently, we have designed and conducted two phase II studies to further evaluate mesothelin as a target of immune response. In both studies, we have found that the induction and maintenance of mesothelin responses is associated with a likelihood of improved survival. However, two new T cell parameters, avidity and the expansion of the T cell repertoire that is specific for mesothelin, are key parameters for predicting prolonged survival. In addition, we have developed a complementary proteomic approach that utilized immunized sera from patients to identify proteins within the vaccine cells against which humoral responses are altered with the vaccine. Utilizing this approach we have identified a panel of proteins against which vaccine induced responses are measured. Preliminarly data demonstrates that one protein in particular, Annexin II, appears to correlate with a vaccine induced survival benefit. This protein appears to be important for pancreatic cancer progression. These new data will be discussed in the context of vaccine development for treatment and prevention of pancreatic cancer.

Keywords: T cells, tumor antigens, pancreatic cancer

350 Targeting Cancer Stem Cells

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Although most hematologic malignancies will respond (often dramatically) to treatment, most patients with these diseases eventually relapse and die of their disease. In fact, it is becoming clear that clinical responses to anticancer therapy often do not translate into improved survivals. The cancer stem cells (CSC) concept is an attractive explanation for the paradox of the relatively poor outcome of cancer patients in the face of high initial response rates. Although generally considered a disease of malignant plasma cells (PC), we found that multiple myeloma (MM) arises from a small population of self-renewing CSC that resemble memory B cells. Not only do these clonotypic B cells circulate in most patients, but they are also resistant to most standard anti-MM agents and thus appear to be responsible for most disease relapses. The MM stem cells resemble their normal counterpart quite closely, such that much of their therapeutic resistance appears to result from mechanisms co-opted from normal stem cells. Reed-Sternberg (RS) cells, the hallmark of Hodgkin lymphoma (HL), are the only blood cells other than PC to occasionally express CD138; this suggests that RS cells represent aberrant PC differentiation, and in fact we found that RS cells express a transcriptional profile similar to normal and malignant PC. Accordingly, we found that HL also appears to arise from CSC that resemble memory B cells, and that these clonotypic B cells circulate in most HL patients even in early stage disease. A characteristic shared with normal stem cells, high expression of aldehyde dehydrogenase (ALDH), is a critical component of our ability to detect circulating CSC in MM and HL.

Although targeting CSC has proved elusive, our data suggest they are still susceptible to novel therapeutic strategies, such as immune-based approaches. Clearly, the allogeneic graft-versus-tumor effect can eliminate hematologic malignancy CSC, but is limited by the toxicity of graft-versus-host disease and the availability of matched donors. About one-third of patients, and most of some ethnic groups, will not have a complete match in unrelated registries. Even when a match can be found, a median of 4 months is required to complete searches; thus, some patients succumb to disease while awaiting identification of a suitable HLA-matched donor. The ability to use haploidentical family members would overcome both of these difficulties. Based on the resistance of normal stem cells to cyclophosphamide (CY) via their high expression of ALDH, the major mechanism of CY inactivation, our group developed high-dose CY post-transplant as a means to induce bidirectional tolerance after allogeneic transplantation. High-dose CY after related haploidentical transplantation results in a toxicity profile similar to that seen with matched sibling donors. Our group also found that T cells present in the bone marrow of MM patients kill MM stem cells, and that regulatory T cells play an important role in down-regulating anti-tumor immunity. Several signaling pathways that are important for the generation and maintenance of normal stem cells during embryonic development or postnatally (eg, Hedgehog or telomerase) also are important for the growth of many cancers. Inhibition of these pathways, even when they are not mutated or overexpressed, produces potent antitumor activity across a range of CSC *in vitro*, possibly because of the key roles these pathways play in stem cell maintenance and growth. We found that induction of CSC terminal differentiation also holds therapeutic promise. Clinical trials targeting CSC immunologically and via these stem cell pathways are currently ongoing.

Assessing the effects of new therapies on CSC will require new clinical paradigms and methodologies that evaluate the effects of therapies on the rare CSC, since traditional response criteria measure tumor bulk and may not reflect changes in rare CSC populations. We believe new paradigms should rely heavily on preclinical modeling, utilize non-traditional trial endpoints (other than clinical response), and evaluate novel preclinical assays that assess the fate of CSC. Accordingly, we recently found that quantifying MM stem cells *in vivo* predicts progression-free survival after MM stem cell directed therapy.

References: Matsui *et al.* Cancer Res. 68:190-7, 2008; Luznik *et al.* Biol Blood Marrow Transplant. 14:641-50, 2008; Huff *et al.* Blood. 107:431-34, 2006; Noonan K *et al.* Cancer Res 65:2026-2034, 2005.

Keywords: cancer stem cells, blood and marrow transplantation, multiple myeloma

351 Exploiting Allogeneic NK Cells in Cancer and Transplantation

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It is becoming increasingly clear that cooperation between both the innate and adaptive arms of the immune system is required to induce a productive immune response. Natural killer (NK) cells participate in this complex response by producing cytokines and by killing targets directly or enhanced by antibody-mediated cellular cytotoxicity (ADCC), functions which are regulated by activating and inhibitory receptors. The tolerance or alloreactivity of allogeneic NK cells is partially determined by their expression of killer immunoglobulin-like receptor (KIR) genes, some of which recognize "self" class I major histocompatibility complex (MHC) molecules. This mechanism explains the limited efficacy seen with autologous NK cell therapy, and is the basis for the selection of donors with KIR that will not be inhibited by recipient class I MHC ligands to exploit their alloreactive potential. Several clinical strategies to predict KIR-based alloreactivity have demonstrated less relapse and better survival in patients with myeloid leukemia. The best results have been reported in settings where T-cells have been depleted from the graft or in vivo, as T-cells may affect the function of reconstituting NK cells. Progress in separation techniques has made adoptive therapy with allogeneic NK cells a viable treatment option. Our main experience has been on treatment options for patients with refractory AML. We tested adoptive transfer of haploidentical peripheral blood (PB) derived NK cells (CD3-depleted lymphopheresis products) without transplantation in over 30 patients and demonstrated a correlation between in vivo NK cell expansion, dependent on a surge in IL-15 and IL-7 induced by lymphodepletion, and complete remission (~25%).

This therapy is limited by: 1) the inability to expand NK cells in most patients, 2) prolonged neutropenia in some patients and 3) inconsistent efficacy. Increasing immunosuppression by the addition of total body irradiation (400 cGy) has resulted in expansion of NK cells in most patients and addition of a haploidentical graft may shorten neutropenia. We are currently performing a phase I dose escalation of bortezomib as part of the preparative regimen to increase target cell sensitivity. Adoptive transfer of allogeneic NK cells is also being tested in breast cancer, ovarian cancer, lymphoma and CLL. Further progress has shown that donor choice may be an important variable. Donor and recipient DNA samples from 209 HLA matched and 239 mismatched T-replete URD transplantations for AML were KIR genotyped using a novel, validated method based on single nucleotide polymorphisms (SNP) analyzed by mass spectrometry. Genotypes were assigned as A/A, denoting presence of two A KIR haplotypes, or B/x denoting presence of one or two B haplotypes. Multivariate models evaluated the effect of donor and recipient KIR genotypes on HCT outcome. Transplantation from B/x donors yielded 30% improvement in the relative risk of relapse free survival compared to A/A donors (RR 0.70 [95% CI 0.55-0.88]; p=.002). This benefit was independent of recipient KIR genotype and of presence of any particular B haplotype-specific KIR. KIR genotyping of prospective donors, in addition to HLA typing, should be performed to identify HLA-matched donors with B KIR haplotypes, which may improve strategies exploring NK cell adoptive transfer. Combined approaches that enhance NK cell killing include the use of monoclonal antibodies to target cells for ADCC, blockade of inhibitory signals, and enhancement of activation signals may ultimately improve the efficacy of NK cell-based therapies.

Keywords: immunotherapy, NK cells, hematopoietic cell transplantation

352 Biology and Therapy of High-Risk Neuroblastoma

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Neuroblastoma is the most common extra cranial solid tumor of childhood, and 45% of patients have aggressive tumors, most of which are metastatic (stage 4) when diagnosed. Previous studies have demonstrated that both intensive cytotoxic therapy with autologous hematopoietic progenitor cell transplant and post-transplant 13-cis-retinoic acid improve outcome. For example, in the recently completed Children's Oncology Group (COG) A3973 study, 49% of patients were event-free survivors and 73% were surviving two years after diagnosis. However, the limit of host tolerance for intensive, non-targeted cytotoxic therapy likely has been reached. The overall **hypothesis** of our Program Project Grant (5 P01 CA81403-09) is that improved survival requires an understanding of the biology of tumor cells, host cells, and of their interactions and the development of therapies that target pathways that are critically important for neuroblastoma growth. The **aims** of the Projects in this PPG are to integrate biologic and preclinical therapeutic research with early phase clinical trials. Project 1: The tumor microenvironment is investigated with emphasis on tumor cell, bone marrow mesenchymal cell, and osteoclast interactions in bone metastasis. Neuroblastoma cells were shown to produce galectin-3 binding protein, which induces mesenchymal cells to secrete IL-6, which, in turn, activates osteoclasts that enhance formation of bone metastasis. A bisphosphonate, zoledronic acid, markedly inhibits osteoclast activity and bone invasion by tumor cells, and this agent was tested for the first time in children by our New Approaches to Neuroblastoma Therapy (NANT) consortium. Project 2: Natural killer (NK) cells alone and with an anti-disialoganglioside antibody (anti-GD2 mAb) were shown to kill drug-sensitive and -resistant neuroblastoma cell lines in vitro. Using neuroblastoma metastatic models in NOD/SCID mice, NK + mAb therapy, if initiated before metastases are detectible by bioluminescent imaging, can cure 65% of mice (minimal residual disease model). However, when disease is detectible, immunotherapy, although increasing survival time, is most effective if combined with other agents (bolus and metronomic cyclophosphamide, bevacizumab, zoledronic acid). Some stage 4 MYCN non-amplified tumors from patients were found to have high levels of gene expression representing immune/inflammatory cells (B cells, macrophages) and cytokines (IL-6, IL-10, TGFβ1), which correlated with <20% progression-free survival. The effects of such a tumor promoting microenvironment on NK + mAb cytotoxicity are being determined. Project 3: The cytotoxic retinoid, fenretinide was shown to increase ceramide induction and tumor cell death. Agents that synergize with fenretinide to further enhance ceramide mediated cytotoxicity have been identified based upon ceramide pathway analyses. A new oral liquid formulation of fenretinide that was developed in collaboration with the NCI RAID program was tested by the NANT consortium and is superior to an earlier capsule formulation with respect to obtaining potentially effective drug levels. Project 4: New strategies targeting tumor and/or normal microenvironment cells are tested in phase I and II trials by the NANT consortium (www.nant.org), which includes 14 pediatric oncology institutions across the US and in Canada and which accrues 55 patients annually. Fourteen clinical trials have been opened through 2007 with four being completed and eight currently active. Approaches that are promising are moved forward in the clinical trial pipeline. For example, targeted radiation from ^{131}I-metaiodobenzylguanidine (MIBG) combined with high dose chemotherapy has been tested in phase I and II trials by the NANT and is now being developed for newly diagnosed patients by the COG. In **summary**, a continuum of integrated pre-clinical and early phase clinical research is providing new insights and therapeutic strategies for achieving our ultimate goal of improving survival for children with high-risk neuroblastoma.

Keywords: neuroblastoma, drugs, biologics

353 Immuno- and Immuno-Gene Therapies for Thoracic Malignancies (Mesothelioma and Metastatic Pleural Disease)

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The treatment of malignant mesothelioma and malignant pleural effusions remains woefully inadequate. It is clear that better treatments for patients with these devastating diseases are urgently needed. Progress in basic tumor immunology has led to many exciting advances in immunotherapy including the identification of tumor antigens, use of specific cytokines to augment anti-tumor responses, antibodies and reagents that can combat tumor-induced immunosuppression, development of tumor vaccines, and ex-vivo generation of cytotoxic effector cells. The underlying hypothesis of our Program Project has been that effective immunotherapy can be achieved using these new technologies and by intervening at multiple points in the generation of an anti-tumor response.

We have approached this goal in a number of ways. Based on a series of preclinical studies in mouse models, we identified interferon-β as particularly effective agent when delivered intra-tumorally by an adenoviral vector. A series of Phase 1 Clinical Trials have been conducted using an adenovirus expressing the cytokine Interferon-β (Ad.IFNβ). More than 25 patients have had pleural catheters placed and Ad.IFNβ instilled as either a single dose (Ref. 1) or two doses separated by one or two weeks. This approach has been well tolerated and feasible. Consistent anti-tumor humoral immune responses have been noted against known and unknown tumor antigens. About one third of the patients have had clinically meaningful responses, i.e. tumor regressions or stable disease.

Preclinical studies have been aimed at augmenting these responses. Significant augmentation has been seen by the addition of COX-2 inhibitors, TGF-β blockade, tumor-associated macrophage activation, and combination with chemotherapy. We are currently planning to conduct a new pilot and feasibility trial combining Ad.IFN with first line chemotherapy in patients with mesothelioma.

We are also developing adoptive immunotherapy for mesothelioma using genetically modified lymphocytes engineered to target the tumor antigen Mesothelin. The strategy is the "T-body" approach that uses genetically reprogrammed, patient-derived lymphocytes transfected with a novel chimeric receptor that contains combinations of the signal transduction domains of 4-1BB (CD137), CD28, and CD3ζ, as well as anti-mesothelin scFv (anti-meso-BB-28-ζ). We have successfully made T-bodies that work well in vitro and in animal models. Current efforts are aimed at appropriate toxicology experiments in preparation for conduct of a Phase 1 Clinical Trial.

Reference: Sterman et al. Clinical Cancer Res 13:4456-4466, 2007.

Funding: PO1 CA066726.

Keywords: immunotherapy, inteferon-beta, adoptive T-cell transfer

354 Mixed Hematopoietic Chimerism After Stem Cell Allografts

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The goals of this Program are to broaden the application and increase success and safety of allogeneic hematopoietic cell transplantation (HCT) after nonmyeloablative conditioning in treating patients with hematologic malignancies. To this end, the grant is composed of two preclinical and two clinical projects. The preclinical Projects 1 and 2 involve a canine model of HCT with a long history of clinical translation. Project 1, which developed the clinical HCT regimens currently used in Projects 3 and 4, will address three major issues in allogeneic HCT. One is to replace the cytotoxic conditioning regimen with biological means of tolerance induction to donor grafts and thereby reduce late regimen-related sequelae. Another is to explore novel ways of preventing graft-vs.-host disease (GVHD) that will avoid the need for and side effects from current long-term postgrafting immunosuppression. The third is to improve eradication of persistent malignancies as seen in a number of patients transplanted under Projects 3 and 4. This third aim will use mixed donor/host hematopoietic chimerism and experimentally-induced leukemia as models of persisting malignant cells and, in collaboration with Project 2, investigate how to enhance graft-vs.-tumor effects without risking GVHD. Project 2 will use genomics approaches to identify canine minor histocompatibility antigens with the goal of discriminating between those antigens whose expression is restricted to hematopoietic cells and those which are ubiquitously expressed. Knowledge generated in this project will increase our understanding of GVHD and graft-vs.-tumor effects. Projects 3 and 4 use allogeneic HCT to treat human patients with advanced hematologic malignancies. The HCT regimen uses truly nonmyeloablative conditioning as evidenced by autologous marrow recovery in those rare patients who reject their grafts. It has minimal early toxicities and, importantly, allows for the purest determination of graft-vs.-tumor effects apart from conditioning and the best determination of GVHD not augmented by regimen-related toxicities. It provides an excellent foundation on which to add disease and disease stage specific modalities, which will include immune manipulations in Project 3 and pharmacological manipulations in Project 4.The public health benefits of the Program are underscored by the fact that, since the clinical introduction of the nonmyeloablative regimen, more than 1,250 patients with various malignant and nonmalignant blood disorders have benefited from allogeneic HCT who otherwise would have been excluded because of age and co-morbidities. This is especially important since median ages at diagnosis of patients with most candidate diseases range from 65 to 70 years, which is beyond the age range of inclusion in conventional myeloablative HCT regimens. Translation from laboratory bench to the clinic is a multi-stage process. First, results of both preclinical and clinical studies are presented and discussed at bi-weekly “Mixed Chimerism” meetings which are attended by faculty, (including statisticians) and postdoctoral fellows, and at an annual one and a half day meeting with collaborators from 15 outside academic centers. This includes discussion of preclinical findings deemed ready for clinical translation. Resulting clinical protocols are presented to faculty of the FHCRC’s Clinical Research Division at weekly Clinical Investigators’ Meetings (CIM). Presentations are followed by criticisms from two CIM reviewers, one Scientific Review Committee (SRC) member, and faculty at large. CIM ends on a closed, advisory vote by faculty, using NIH ranking (1-5). One week later the protocol (revised in response to CIM criticism) is reviewed by SRC which is composed of 9 senior faculty, including respective SRC reviewers. Discussion centers on scientific aspects of each protocol, its place in the scientific mission of the Center, its impact on other ongoing clinical research protocols (priority), and its feasibility (patient accrual). If the closed SRC vote is “yes”, the protocol will go to the FHCRC’s IRB after appropriate correction. If the vote is “no”, the protocol goes back to the drawing board. Once IRB approved, the protocol is reviewed by Standard Practice and Pharmacy committees and then is opened and conducted with assistance by research nurses, clinic staff and attending physicians of the Clinical Research Division. Each protocol undergoes annual reviews by IRB, Protocol Data Monitoring Committee (PDMC) and, if multicenter, also a Data Safety Monitoring Board (DSMB) which meets semi-annually. If these committees raise concerns, protocols are referred to SRC for further review.

Keywords: allogeneic HCT, graft-versus-tumor effect, hematologic malignancies

355 Development of an Autoantibody-Based Early Detection Test of Ovarian Cancer Using Protein Microarrays

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Cancer patients develop antitumor immune responses, both humoral and cellular, against antigens expressed by their tumors. Low-throughput antigen cloning has been used to identify a number of immunogenic tumor antigens, but with limited utility for diagnostics or therapeutic vaccines. A novel approach to the identification of diagnostic antigens that utilizes a combination of high-throughput selection and protein-microarray-based serological detection of complex panels of antigens that are indicative of the presence of cancer, is described herein, and should lead to a great diversity of vaccine candidates. This technology exploits the immune system as a biosensor to diagnose the presence of cancer through serum testing, and the repertoire of antigen biomarkers identified can be employed in the future as therapeutic targets. In order to developing a more effective set of antigen biomarkers for autoantibody immunoassays on protein microarrays we have been working to expand of set of tumor antigen clones.

Biopanning of 4 gene libraries using 8 different OVCA sera: We prepared 4 T7 Phage Display cDNA libraries and biopanned them with 8 different patient sera. Each library has a separate DNA linker for barcoding purposes so that we can identify the origin of each selected cDNA clone. This is a new process that we developed. Antigen biomarker clones were picked and screened to lower the total number of biomarkers for further studies (attribute reduction in the machine learning vernacular) We tested 2800 antigen clones on a small set of patients and used a variety of informatics tools to select the best markers. We split up the clones into 2 sets to provide better separation between spots (quintuplicate) on the array (see diagram). Therefore sets 1 and 2 were each tested on two arrays per patient serum. The clones from each biopanning level 4 were used if they exhibited a strong of the IgG binding signal in the self-binding chip and at least two other patients' sera. To reduce the number of clones such that they could be spotted on a single microarray for the large validation sets. We used a series of approaches based on the discussion from a series of team meetings in which there was interest in developing novel informatics methods for "biomarker choice". The validation of the resulting markers will allow us to determine the best approach to marker choice.

1) Bootstrapping method combined with an ROC analysis. **2)** Parametric test (T-test). **3)** Non-parametric test (U-test: analysis on ranks; less sensitive to outliers). The union of the top 600 clones from all the 3 methods is 776 indicating that among the 2800 antigens many were found to be good markers by all methods. Among these 776 markers 432 were chosen as top clones by all 3 methods. A number of negative controls were also chosen.

Validation Serum Sets: We are using a set protein microarrays to validate the above 1010 selected markers including those 166+65 antigens identified in the early phase of this project which after DNA sequencing was 133 different antigens. The validation sets are listed below and will involve the analysis of more than 1800 blood sera on protein microchip arrays:

Clone Selection: We have developed new ways to find biomarkers for diagnostic tests for ovarian cancer. Below is a table that summarizes the methods used to identify biomarkers that distinguish early stage, Stage I, ovarian cancer from benign gynecological diseases. Two statistical tests were used, a t-test and ranking by receiver-operator characteristics. We concentrated on the biomarkers that overlapped in both methods and any that were identified in multiple experiments and we identified a panel of 92 ovarian cancer specific markers. The sensitivity of the panel of 92 biomarkers was 75% which indicates the test using the current technology would identify three quarters of all serous ovarian cancer even at early stage. The specificity is outstanding at 95%. This experiment involved serum samples from 155 malignant serous cancer patients' serum and 288 healthy controls. None of these women's serum samples were used in previous steps of biomarkers discovery.

Keywords: early detection, autoantibodies, protein microarrays

356 Clinical and Serologic Markers of Benefit in Metastatic Melanoma Treated With Combination Immunotherapy of IFNα -2b and Anti-CTLA4 Blockade: Preliminary Data

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High dose IFNα-2b (HDI) and anti-CTLA4 blockade with tremelimumab are two immunomodulatory therapeutic approaches that demonstrate activity in advanced melanoma. We hypothesized that the combination of these agents would induce more potent antitumor immunity and antitumor responses.

Tremelimumab was given at 15 mg/kg IV per course (12 weeks). HDI was given concurrently starting with IV induction at 20 MU/m2 IV, 5 days/week, for 4 weeks followed by maintenance with 10 MU/m2 SC three times a week , for 8 weeks per course. From course 2 onward, HDI was given only SC at 10 MU/m2 TIW. A 2-stage design was adopted. Clinical and serologic (ELISA; DiaSorin, Stillwater, USA) markers of autoimmunity and multiplex analysis (SearchLight Proteome Arrays, Boston, MA) of serum protein levels (IL1a, IL1b, IL6, TNFa, MIP1b, IFNa, EGF, IL2R, MIP1a, IP10, VEGF, HGF) were measured at baseline and during therapy. Two preliminary analyses were conducted: 1) whether biomarkers were modulated by IFNα + anti-CTLA-4 and 2) whether biomarkers exhibited significant differences between clinically benefited patients (PR + SD) and 2 more without benefit (PD).

Sixteen patients (10 male, 6 female), age 32-75 (median 55) have been enrolled to date. All had AJCC stage IV melanoma (4 M1a, 2 M1b, 10 M1c) and all had previously received therapy for metastatic disease (range 1-5 prior regimens). Two patients had previously treated stable brain metastases. A total of 27 courses have been administered (median of 1 course per patient; 3 patients continue on therapy). The frequency of grade 3/4 toxicities does not exceed experience with the FDA-approved HDI regimen alone. The overall response rate is 19% (3 partial responses lasting 6+, 9+ & 9 months). Responses were seen in both M1a (1) and M1c (2) disease. Two of the PRs were associated with clinical autoimmune manifestations (autoimmune colitis and marked vitiligo). Six patients have stable disease lasting 1.5 – 10+ months and 4 have had progression. Among 7 patients with PD, 1 had evidence of autoimmunity compared to 8 out of 9 patients with SD or PR (p=0.009; Fishers' exact test). IFNα + anti-CTLA-4 therapy resulted in a significant increase of serum levels of the antiangiogenic IFN-gamma inducible protein 10 (IP-10; p_2=0.002) and the marker of T-lymphocyte activity IL2 receptor [IL2R; p_2=0.003].

Development of vitiligo and autoantibodies is significantly associated with therapeutic benefit following IFNα-2b and anti-CTLA-4 combination biotherapy. Serum levels of IP-10 and IL2R are significantly modulated by this therapy. This study has now proceeded into a second stage in which 21 more patients will be treated.

Keywords: high-dose IFNαb and Anti-CTLA4 blockade antibody, immunotherapy, combination

357 Development of Saline Enhanced Radiofrequency Ablation for Treatment of Liver Tumors

Michael G. Curley, Rich Hanna, Fredrick Kim, Patrick S. Hamilton

E.P., Limited

Ablation therapy using radiofrequency (RF) energy delivered to a tumor to cause hyperthermia has been used to treat small tumors. Conventional RF ablation has difficulty heating tumors larger than 3 cm diameter because most energy production occurs within 4 mm of the RF electrode and tissue conducts heat poorly to distal tissues. Saline Enhanced RF Ablation (SERF™ Ablation) uses the simultaneous injection of warm saline through the ablation electrode into the tumor tissue to convect the thermal energy deep into tissue. SERF Ablation increases the effective heat transfer coefficient of tissue by a factor of 20. Preclinical testing shows that SERF Ablation can ablate 10 cm diameter tumors in 5 minutes. This therapy will be useful to treat large, solid tumors such as those associated with primary and metastatic liver cancer.

The creation of the SERF Ablation modality has resulted in an integrated system containing the radiofrequency generator, a syringe pump driven by a linear actuator, saline heater electronics and a simplified user interface tied to the system controller. The system is housed in a dedicated cabinet manufactured from a combination of welded sheet metal and thermoformed panels. Figure 1 shows the completed system. The porous radiofrequency electrode is machined into a stainless steel needle using electrical discharge machinery. The electrode area is defined by coating the remainder of the needle with a perfluoropolymer. The electrical and saline connections are made to the needle within an injection-molded handle. Figure 2 shows the completed needle design. The needle is fabricated in two clean rooms, class 100 for the fluid path, and class 10,000 for final assembly and packaging.

FDA clearance will be obtained through the 510(k) process first for the indication of ablation of soft tissue. Bench testing shows the system is compliant with EN60601-1, “International Standard for Medical Electrical Equipment,” 60601-1-1, “General Requirements for Safety,” 60601-1-2, “International Standard for EMC Testing of Medical Electrical Equipment”, 60601-1-4, “Collateral Standard: Programmable electrical medical systems,” 60601-2-2, “Particular Requirements for the Safety of High Frequency Surgical Equipment,” and EN60601-2-24:1998, “Particular requirements for the safety of infusion pumps and controllers.” The needle electrode is shown to be compatible with ISO 10993 biocompatibility standards. Preclinical testing in a porcine liver model will complete the verification and validation of the system prior to the 510(k) application.

Figure 1: SERF™ Ablation System

Figure 2: SERF™ Ablation Needle

Keywords: radiofrequency ablation, liver cancer, hyperthermia

358 Development and Characterization of a Magnetic Biopsy Needle and Magnetic Nanoparticles for Sensitive Detection of Cancer Cells in the Bone Marrow

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Acute leukemia is a hematopoietic malignancy for which the accurate measurement of minimal residual disease is key to improving treatment and patient survival. Bone marrow aspiration and light microscopy are the current standard of care for detecting residual disease, however these approaches cannot reliably discriminate less than 5% blast cells. To increase the sensitivity of targeted sampling a “magnetic biopsy needle” has been developed. This device utilizes antibody-conjugated superparamagnetic iron oxide nanoparticles targeted against acute leukemia antigens or rare cancer cells to collect these cells from the marrow. The device has been tested with high and minimally CD34-expressing cell lines incubated with anti-CD34-conjugated nanoparticles using newly acquired bone marrow aspirates.

The superparamagnetic magnetite nanoparticles were characterized by Superconducting Quantum Interference Device (SQUID)-relaxometry, susceptometry, and TEM to determine their size and magnetic moments. Theoretical calculations were made to determine collection efficiencies and times for these particles in the marrow media.

To assess nanoparticle-cell binding, three separate approaches utilizing microscopy, SQUID magnetometry, and *in vitro* magnetic needle extraction were employed. Using appropriate controls, CD34- conjugated nanoparticles were shown to preferentially bind high CD34- expressing cell lines as identified with Prussian blue stained microscopy. This was confirmed by SQUID sensor measurements of the magnetic moments of the extracted nanoparticle labeled cancer cells. The magnetic needle exhibited the capacity to isolate CD34-positive cell lines from non-malignant cells derived from peripheral blood. Analysis of the binding of CD34-conjugated nanoparticles to U937 leukemia cells revealed 60,000 nanoparticles per cell, which were collected from whole blood using a prototype magnetic biopsy needle, with a capture efficiency of >65% from a 750 µl sample volume in 1 minute. The magnetic needle also showed the capability to identify blast cells that were spiked into normal blood at concentrations below those normally found in remission marrow samples. These data indicate that this device can increase the sensitivity of bone marrow biopsy using antigen-targeted magnetic nanoparticles, in combination with the magnetic needle, for the evaluation of minimal residual disease in acute leukemias and the detection of rare cancer cells in the marrow. Currently, the device is used for in-vitro measurements in extracted marrow but in-vivo measurements are also envisioned.

Keywords: hematopoietic, magnetic nanoparticle, biopsy needle

359 Optical Technologies for Cervical Neoplasia

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We have formed a multidisciplinary group of clinicians, scientists, statisticians, epidemiologists, molecular biologists, behavioral scientists, and decision scientists to develop and assess optical technologies for the developed and developing world using the technology assessment paradigm of Littenberg: biologic plausibility, technical efficacy, clinical effectiveness, patient and provider outcomes, and cost-effectiveness, also called societal outcomes. Our group is experienced in performing clinical trials in the U.S., Canada, and Nigeria. We are leveraging funding from several sources to accomplish our goals. In the previous eight years of funding we have: held 14 conferences; written 150 manuscripts and 100 abstracts; and trained 100 undergraduate students, 50 masters' level students, 60 PhD students, and 20 post-doctoral fellows. We have evaluated quantitative cytology in Phase II trials in 1850 patients at three clinical sites, quantitative pathology of the cervix in Phase II trials of 1850 patients with 3765 biopsies at three clinical sites, fluorescence and reflectance spectroscopy using a point probe that measures 2 mm of tissue in Phase II trials in 1850 patients with 3765 biopsies at three clinical sites, and fluorescence and reflectance spectroscopy using a multi-spectral digital colposcopic approach that images the entire cervix in pilot trials (100 patients in five clinical sites). Behavioral research on the point probe and multispectral digital colposcope was conducted along with the clinical trials. Methodological work supported the trials. Similarly, data on accuracy and cost-effectiveness analyses of these four technologies are now ready and methodology reports have guided the direction of analysis.

Cervical cancer is the second most common cancer in women worldwide and the leading cause of cancer mortality for women in developing countries. When precancerous lesions are detected early they are easily treatable and cause no decrease in survival. Cervical cancer is a devastating disease and the treatment for more advanced stages is morbid, expensive, and ineffective. In the developed world we have good screening and detection programs, but these are expensive and require a well-developed infrastructure. In the developing world, where resources for screening are not available, many young women die of a preventable disease. Optical technologies provide a solution to these problems. Optical measurement of tissue provides quantitative information that can be analyzed, instantaneously producing an objective diagnosis even in the hands of a non-expert operator. Devices to make these measurements have become inexpensive, robust, and portable because of advances in related fields of engineering.

The innovative aspects of this program project are four-fold: 1) the project uses the cervix, a small and accessible organ for which the dysplasia-carcinoma sequence is well-understood as the basis for examining emerging optical technologies; 2) optical spectroscopy and quantitative cytologic and histopathologic analyses are evaluated for biological plausibility, effectiveness, acceptability, and cost-effectiveness in both the screening and diagnostic settings; 3) both technologies will have broad applications to other organ sites such as the oral cavity and lung, the digestive tract, the bladder, and skin; and 4) we have assembled a research network that is multidisciplinary, synergistic, and dedicated.

Keywords: cervical cancer, dysplasia-carcinoma, optical spectroscopy and quantitative cytologic and histopathologic analyses

360 MR-Guided Thermal Therapies and Surgeries: A Brief History of the Translation of Innovative Technologies to Ground-Breaking Clinical Practice

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Interventional Magnetic Resonance (MR) Imaging (MRI)—MR image-guided therapies (IGT) and image-guided surgeries (IGS)—has evolved dramatically in the past 20 years at Brigham and Women's Hospital , Harvard Medical School Image-Guided Therapy Program. The identification of the possibility of MRI use for MRI-guided thermal therapies was a key breakthrough for IGT [1] triggered by the discovery earlier of the thermal sensitivity of MRI [1] .From 1994 till the end of 2006 more than 3100 MR-guided IGT and IGS procedures were performed at BWH using the 0.5T GE Signa SP vertical configuration open MR scanner [2)]. Clinical firsts include: the first MR-guided open neurosurgery, prostate brachytherapy; first MR-guided ENT endoscopy; first laser ablation of liver tumors; first liver cryotherapy. MRI's capabilities of exquisite tissue definition / characterization, high spatial resolution, multi-planar or 3D acquisition, dynamic imaging, vascular depiction, functional characterization, and ability to elucidate anatomy and pathology are ideal for the needs of IGT and IGS procedures that include: planning, probe/needle guidance, probe tracking and navigation, monitoring of the procedure's progress (and extent of the thermal front for thermal therapies), control of the procedure, and final assessment of IGT and IGS effectiveness [3].

In 2008 BWH is starting construction of its AMIGO (Advanced Multi-modality Image-Guided Operating) suite, the next generation of clinical facilities focused on IGT and IGS. The AMIGO Facility is to be constructed by BWH in cooperation with GE Healthcare Inc. and an NIH High-End Instrumentation grant (S10-RR019902) will be composed of a 16 channel cylindrical configuration 3T GE MR scanner, with MRgFUS capability, and a GE PET/CT scanner (using short-lived isotopes produced by our own in-hospital cyclotron) surrounding a state-of-the-art operating room with multiple large-screen monitors for viewing images and IGT/IGS-specific visualizations of images. These modalities will be accompanied by an optical imager for molecular imaging , diagnostic 3D US, x-ray fluoroscopy, and - for real-time in-OR tumor characterization - a mass spectrometer and high resolution NMR spectrometer. The aims of our current funding period of P01-CA67165 grant include a focus on applications-related projects: integrated intraoperative guidance for neurosurgery; advanced MRgFUS applications; image-based navigation for interventional robotic devices; implementation of parallel MRI data acquisition techniques; and MR-guided interventions (MRgFUS) in the prostate. Basic IGT and IGS technology is now being developed in our synergistic National Center for Image-Guided Therapy (NCIGT) [http://www.ncigt.org]

The future of MR-guided IGT and IGS centers on the addition in each procedure of cellular-level chemical, functional and physiological information to multi-modality radiological imaging and functional information. We will see increasingly more coupled use of multiple radiological, laparascopic, endoscopic, spectroscopic and microscopic modalities in the course of a procedure in order to provide for more accurate identification of tumor margins, for intra-procedure tumor cell analysis, and for monitoring the progress of therapies. MRI, x-ray CT, Nuclear Medicine and Optical Physics will enjoy a freshly renewed intimate relationship. New imaging technology will aspire to dynamically acquire and use high quality, high resolution, artifact-free 3D images from moving organs. Both surgeries and therapies will come to rely heavily on intra-procedure 3D visualization software, and a greater range of software tools for monitoring and control that will aim to increase the capabilities and efficiency of the interventionalist while ensuring patient safety.

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Keywords: interventional MRI, thermal ablations, image-guided therapy

361 Multicenter Selective Lymphadenectomy Trials for Melanoma

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We introduced sentinel node biopsy (SNB) in the early 1990s to resolve the long-standing controversy over elective lymph node dissection (LND) versus watchful waiting for patients with primary cutaneous melanoma.[1] Early removal of regional node metastases improves survival, but only 20% of patients with clinically normal regional nodes have histopathologic evidence of nodal involvement. SNB is a minimally invasive technique to identify these patients without subjecting all patients to the potential morbidity of LND.

SNB identifies and removes the first tumor-draining lymph nodes (sentinel nodes [SNs]) on the afferent lymphatic pathway. Because focused histopathologic analysis of the SN accurately predicts the tumor status of all nodes in the drainage basin, LND is undertaken only in patients with SN metastasis. Studies have validated the SN concept not only for melanoma[2] but also for many other solid tumors that drain via the lymphatics.[3]

SNB is a multidisciplinary technique: the nuclear medicine physician uses preoperative lymphoscintigraphy to identify the lymphatic drainage basin; the surgeon uses intraoperative lymphatic mapping to identify and remove the SN in this basin; the pathologist uses immunohistochemical and molecular techniques to examine the SN for tumor. To determine the feasibility of multidisciplinary SNB for widespread use, in 1994 we began the international Multicenter Selective Lymphadenectomy Trial (MSLT: A Clinical Study of Wide Excision Alone versus Wide Excision with Intraoperative Lymphatic Mapping and Selective Lymph Node Dissection in the Treatment of Patients with Cutaneous Invasive Melanoma). Accrual was completed in March 2002, with 17 centers randomizing 2001 patients. MSLT data have confirmed the accuracy and minimal morbidity of SNB.[4] Interim analysis also showed that SNB-based staging of intermediate-thickness (1.2 to 3.5 mm) primary melanomas provides important prognostic information and identifies patients with nodal metastases whose survival can be prolonged by immediate LND.[5]

MSLT data indicate that most (70%–80%) melanoma patients with SN micrometastases have no other tumor-involved nodes. Is LND still necessary? This question will be answered by the second MSLT (MSLT-II: A Phase III Multicenter Randomized Trial of Sentinel Lymphadenectomy and Complete Lymph Node Dissection versus Sentinel Lymphadenectomy Alone in Cutaneous Melanoma Patients with Molecular or Histopathological Evidence of Metastases in the Sentinel Node). Of the 4,200 patients to be accrued at melanoma centers around the world, 1925 patients with a tumor-positive SN will be randomized to LND or to nodal observation with serial ultrasound of the SN basin. MSLT-II was initiated in 2004; currently 2102 patients have been screened, 43 sites in the US, Canada, Israel, Europe and Australia are enrolling patients, and 588 patients have been randomized. Of the randomized patients, 451 have histopathologic evidence of SN metastasis by hematoxylin/eosin or immunohistochemical staining, and 137 have molecular evidence of SN metastasis by real-time quantitative RT-PCR assay with a panel of multiple markers.

Funding: NCI P01 CA029605.

Reference: [1]*Arch Surg* 1992;127:392-9. [2]*Ann Surg* 1994;220:759-67. [3]*Cancer J Sci Am* 1998;4:351-8. [4]*Ann Surg* 2005;242:302-11. [5]*N Engl J Med* 2006;355:1307-17

Keywords: melanoma, sentinel node biopsy, phase III trial

362 MRI-Guided Breast Biopsy System

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[1]Robin Medical, Inc.; [2]Norton Healthcare

Background and Significance: The role of diagnostic breast MRI for early detection of breast cancer in high-risk subjects is well established, and as a result there is a surge in the use of screening breast MRI by the general population. However, once a suspected lesion is found, the required biopsy is done by guidance through other modalities (e.g. ultrasound) or outside the bore of the MRI using stereotactic guidance technique. Solutions under development for in-bore breast biopsy are based on robotic systems which are expensive, require a long regulatory clearance pathway, and will require substantial setup time for system registration for each patient, unless a dedicated MRI scanner for breast biopsy is used. We are developing a self-contained add-on system that is similar in shape to standard breast coil, does not require any setup process before each procedure, and can be used on any MRI scanner. This will enable rapid adoption of the technique by clinical users and will provide a superior guidance solution compared with current systems or systems under development.

Methodology: Two features need to be added to standard MRI scanner to facilitate in-bore MRI-guided interventions: tracking of the interventional device and remote operation of the intervention. Device tracking is done by the EndoScout tracking system that uses the gradient fields of the scanner for 6-degrees of freedom tracking of small passive sensors (the EndoScout is FDA cleared and is used clinically on open MRI scanners). To enable remote operation of the intervention, we have developed a manipulator that is integrated into a customized breast imaging coil (Figure 1). The manipulator enables the operator to align a coaxial needle to the target and to insert it into the target, all under real-time MRI (Figure 2). Then the biopsy itself is done out of the scanner by using the outer part of the coaxial needle as a needle guide to the target. Technical testing of the system has been conducted on a Signa-SP open MRI scanner (GE Healthcare) at the Norton Hospital in Louisville, KY, by a surgeon experienced with image-guided breast biopsy (AC). Needle insertions were done into chicken breast phantoms with olives as the target to the biopsy.

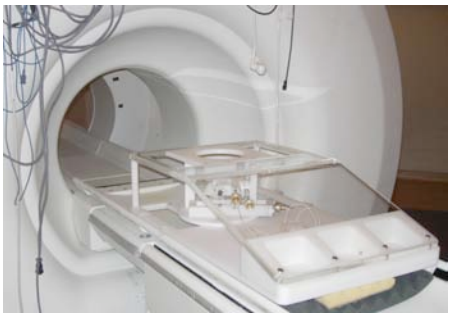

Figure 1: Integrated breast coil and manipulator

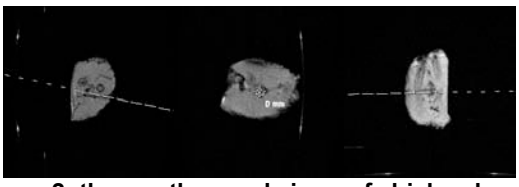

Figure 2: three orthogonal views of chicken breast with olive target and tracking annotation

Results: The integrated breast imaging coil and manipulator for needle alignment and insertion has the general architecture of a standard imaging breast coil, except for the use of a single breast coil. This architecture enables access to the breast from any direction, and provides higher image quality as the breast is positioned at the center of the scanner. Since most of the biopsies are done on a single breast, following an earlier diagnostic scan, this configuration is clinically practical. The operator of the system, who is experienced with breast biopsy procedure with direct access to the breast and manual operation of the biopsy device, liked the performance of the system and provided significant feedback that resulted with design changes to account for the operator needs. During initial tests the success rate for getting a biopsy from the olive (extracted sample contained olive) was 68%. **Conclusions:** The first generation of an integrated breast coil and device manipulator has been tested successfully by biopsy of phantoms emulating breast. Feedback from the operator resulted in design changes. The second generation system, now being manufactured, will pass a similar set of technical tests before it is tested clinically on open scanner, initially, and closed bore scanners, eventually.

Keywords: image-guided intervention, breast cancer, tracking and navigation

363 Clinical Utility of Image-Guided Liver Surgery

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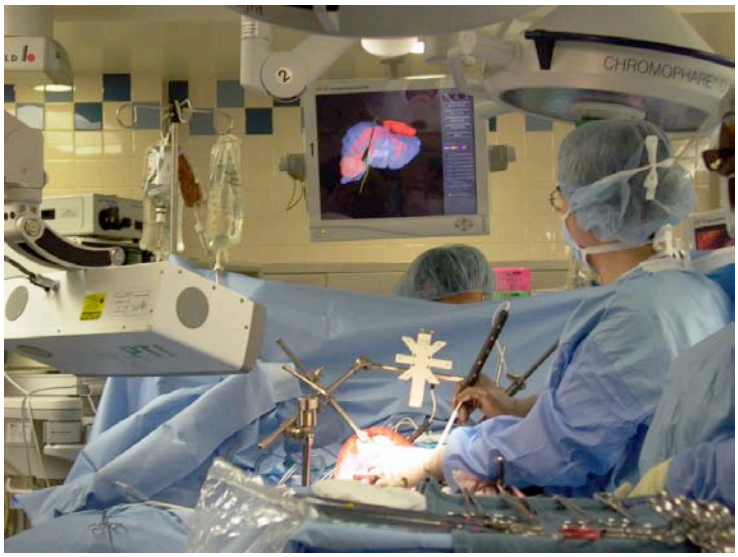
Because it performs complex vascular functions involved in the storage and filtration of the blood, the liver is susceptible to the development of both primary and metastatic solid tumor cancers. When this highly vascularized organ is affected by cancer or other abnormalities, surgical procedures are particularly challenging. Bleeding, infection and adhesions are common complications in open liver surgery, although it remains the gold standard approach for cancer treatment and provides the best chance for long-term survival.

The SurgiSight *LINASYS* (*LI*ver *NA*vigation *SY*stem) was commercially developed at Pathfinder Therapeutics, Inc. (PTI) following a technology transfer of academic research conducted at Vanderbilt University and Washington University in St. Louis. This image-guided liver surgery system enables surgeons to navigate within the liver during surgery using information-rich preoperative CT or MR images and provides a more detailed perspective than that which is currently available. The *LINASYS* is essentially a "global positioning" system (GPS) that allows surgeon to visualize, plan and execute any open liver surgery using both 2-D and 3-D images. Through the use of a multi-capable adapter, ablation devices, electrocautery and ultrasonic cutters can be tracked in real time and their location accurately indicated on the preoperative medical images. The photograph to the right shows the device utilized in the clinical setting.



PTI created several *LINASYS* prototype devices for testing and preliminary clinical evaluation, and the device received FDA 510(k) clearance in December 2007 (K071063). The company is now evaluating the *LINASYS* and another image processing software device for preoperative surgical planning in a three-site clinical trial for those patients suffering from liver cancer. The goal is to establish open image-guided liver resection as the standard of care in treatment of liver cancer. The most thorough and objective assessment to accomplish this goal would be to compare the surgeon's planned resection prior to operation to the resection actually accomplished in the operating room. By comparing the predicted residual liver volume (RLV, calculated from the preoperative imaging and based on the planned resection) to the actual RLV (measured from early postoperative imaging), the adequacy of the resection can be quantified. Calculation of residual liver volume (RLV, also known as future liver remnant volume) is now performed routinely to help stratify the risk of postoperative liver failure in patients undergoing major resection. In the present study, measurement of preoperative RLV will be done primarily for comparison with the measured postoperative RLV, and the correlation between these values will represent the primary endpoint of the study. The hope is that this study will help set benchmarks that allow clinicians to better evaluate the clinical utility of image-guided surgery in the treatment of not only liver but other colorectal and gastrointestinal cancers.

Keywords: image-guided surgery, liver cancer, medical device

364 Long-Term Survival Following Endoscopic and Surgical Treatment of Mucosal (T1a) Esophageal Adenocarcinoma in Barrett's Esophagus

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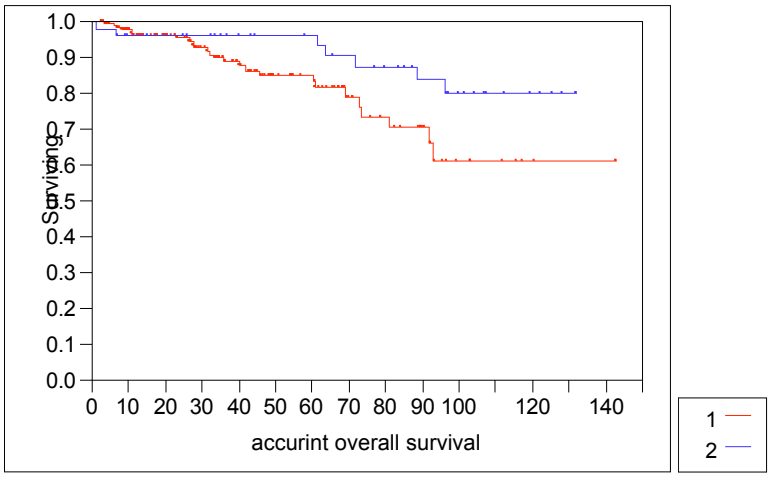
Background and Aims: Endoscopic therapy is emerging as an alternative to surgical therapy in patients with mucosal (T1a) esophageal adenocarcinoma given the low likelihood of lymph nodal metastasis. Limited information is currently available on the long term outcomes of patients treated endoscopically, especially in comparison to a surgically treated group. We aimed to compare the long term overall survival of cohorts of patients with mucosal esophageal ACA treated endoscopically with those treated surgically.

Methods: Records of all patients treated for esophageal adenocarcinoma at Mayo Clinic Rochester between 1995 and 2007 were reviewed. All patients with mucosal (T1a) adenocarcinoma were identified. Histology was assessed by pathologists with expertise in Barrett's esophagus associated neoplasia. Patients were divided into an ENDO (treated endoscopically with EMR with or without PDT) group and a SURG (treated by esophagectomy) group. Patients in the ENDO group were treated using EMR (performed with the Olympus EMR cap or the Wilson Cook Duette kit) and photodynamic therapy (PDT) using previously described standard methods. Data was abstracted from a prospectively maintained database for those in the ENDO group and by review of medical records for the SURG group. Esophagectomy was performed by either transhiatal or trans-thoracic approaches by experienced surgeons. Vital status and death date information was queried using an institutionally approved internet research and location service. Statistical analysis was performed using Kaplan Meier curves and Cox proportional hazards ratios.

Results: 222 patients were identified of which 172 (77%) were in the ENDO group and 50 (23%) were in the SURG group. Patients in the ENDO group were older (mean age 71) than those in the SURG group (mean age 66 years) (p=0.015). Gender distribution was comparable. Mean follow up in the ENDO group was 42 months (SEM 2.5) and 76 months (SEM 4.7) in the SURG group. Cumulative mortality was 14.5% (31/172) in the ENDO group which was comparable to the SURG group (14%, 7/50) (p=0.92). Overall survival was also comparable using the Kaplan Meier method (see Figure 1 below). Using Cox proportional hazard modelling, age was the only significant predictor of survival after adjusting for gender as well as treatment modality. Treatment modality (endoscopic versus surgical) was not a significant predictor of survival on multivariable analysis. Biomarkers including FISH, IHC, and expression arrays are being evaluated as part of a prospective cohort.

Conclusion: Overall mortality and long-term survival in patients with mucosal adenocarcinoma arising in BE, when treated endoscopically (with EMR with or without PDT) appears to be comparable to that of patients treated with esophagectomy. Biomarkers appear to be promising in determining the results of therapy.



Wilcoxon rank sum test p=0.15

Keywords: esophageal adenocarcinoma, long term survival, biomarkers

365 Image-Guided Stereotactic Radiosurgery of Breast Cancer

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One in eight women develops breast cancer in her lifetime. Awareness and screening have resulted in the majority being diagnosed in early-stage, for which the standard of treatment is breast conservation therapy (BCT): lumpectomy followed by 5-7 weeks of radiotherapy. Emotionally and cosmetically, BCT is better than mastectomy. However, patients suffer a lot to keep their breast. ~30% lumpectomies require re-excision because of the lack of image guidance; the protracted daily radiotherapy creates fatigue and is emotionally draining; and the cost, multiples that of mastectomy, is financially challenging for many families. Nevertheless, no significant advance has been made in BCT for decades.

We believe that radiosurgery, a painless, noninvasive procedure, is ideal for breast cancer. With pin-point precision, radiosurgery allows surgical style eradication of tumors without a knife and has been proven effective for all tumors at a location where a high dose of radiation can be safely and precisely delivered. Radiosurgery has never been applied to breast, because there is no radiosurgery device for breast and no method to localize the tumor with pin-point accuracy. These obstacles have been solved by several inventions (patent pending) that we are devoted to develop in this research proposal: 1) a rigid but comfortable cup which locks to the table with mild negative pressure immobilizing the breast; and 2) a irradiation device that focuses high doses of radiation on the tumor, thereby eliminating surgery, and gives just sufficient dose to sterilize potential residual tumor cells surrounding the tumor, thereby eliminating the need for post-operative radiotherapy.

The development and application of the radiosurgery system will bring immediate relief to the suffering of many women. A woman with early stage breast cancer would complete first line of therapy (currently surgery and radiation) in less than an hour rather then months, comfortably listening to iPod, with no anesthesia, no needle or knife, no pain, and no scars.

Keywords: radiation, surgical device, breast cancer

366 Genetics of Cognitive Decline Post Cancer Chemotherapy

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Breast cancer treatments, including chemotherapy and endocrine therapy, have been associated with cognitive decline in a subgroup of survivors, suggesting that genetic factors may increase risk for post-treatment cognitive changes. Our earlier research demonstrated that long-term survivors of breast cancer and lymphoma who had been treated with chemotherapy and were APOE 4 positive scored significantly lower in certain domains of cognitive functioning as compared to survivors with other forms of the allele. The current study examined the relationships between changes in cognitive functioning and genetic polymorphisms in breast cancer patients evaluated prior to and following adjuvant chemotherapy. Matched groups of breast cancer patients not exposed to chemotherapy and healthy controls were evaluated at similar intervals. A genotyping array (Affymetrix MIP) was designed to assess candidate SNPs in the following pathways: DNA repair, plasticity/repair and growth factors, blood brain barrier integrity, neurodegeneration, neuroinflammation, neurotransmission and receptors, cerebrovascular and metabolic/endocrine factors, and the molecular substrates of cognition and memory/long term potentiation. Initial analysis has suggested associations between chemotherapy-induced cognitive decline and genes related to DNA repair (MRE11A, MAG) and plasticity / repair (APOE). Further analyses will focus on genes that regulate blood brain barrier efficiency (MDR1).

Understanding genetic factors that increase risk for cognitive changes associated with cancer treatments may lead to an understanding of the molecular mechanisms of cognitive changes in breast cancer survivors and to the development of targeted treatments to prevent or reduce the negative impact of these cognitive changes.

Keywords: chemotherapy-induced cognitive decline, DNA repair genes, APOE

367 The Relationship Between Sleep, Fatigue, and Chemobrain in Women With Breast Cancer

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Patients treated with chemotherapy report decreased cognitive functioning before and during chemotherapy, as well as for some years after the end of therapy. Understanding the cause of the cognitive deficits is critical as these patients require increased care, experience impaired decision making ability and decreased quality of life, and express concern about their ability to maintain employment. Fatigue and problems sleeping are also commonly reported as major concerns by people undergoing chemotherapy, particularly those being treated for breast cancer. Yet whether chemobrain reflects neurocognitive changes resulting from chemotherapy, or might be secondary to fatigue and/or sleep and circadian rhythm problems, has not yet been explored. We present interim data from an ongoing study in which we are examining the role of sleep disturbances and fatigue, as well as hormonal changes, anxiety and depression, as potential proximal causes of cognitive impairment in women undergoing chemotherapy for breast cancer.

Participants in our study are women with breast cancer recruited before the start of chemotherapy, and tested pre-treatment, at the end of four cycles of chemotherapy and one year later. Non-cancer controls are also recruited and tested using one-to-one matching on age, demographic and socioeconomic factors. At all three time points, data are collected on subjective (self-report) measures of fatigue, sleep quality, depression, anxiety, menopausal symptoms and quality of life and a complete neuropsychological test battery is administered. Sleep/wake activity is also objectively recorded with actigraphy and blood is collected to assess inflammatory markers.

Interim analyses showed that patients (n=21), compared to controls (n=21), reported significantly more fatigue ($p<0.001$), anxiety ($p<0.001$), depressive symptoms ($p<0.001$) and worse sleep quality ($p=0.029$) even before starting chemotherapy. Patients also reported more deterioration from pre- to post-chemotherapy (approximately 12 weeks), compared to controls, in sleep quality ($p=0.015$), depressive symptoms ($p=0.0002$), anxiety ($p=0.004$) and cognitive functioning ($p=0.024$). A linear regression analyses was computed to determine what factors, after controlling for baseline cognitive levels, predict a worsening of cognitive function over four weeks of chemotherapy. Interim results suggested that only a worsening of fatigue was significantly related to a decrease in cognitive function ($p=0.027$) with the model explaining 19% of the variance.

Although based on interim analyses from an ongoing study, these preliminary data suggested that fatigue is related to decrements in cognition. If these results are supported with larger samples, intervention studies aimed at improving fatigue for patients undergoing chemotherapy should be considered.

Supported by NCI CA112035.

Keywords: sleep, fatigue, chemobrain

368 Psychological Intervention for Women With Breast Cancer

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Background. Psychological and behavioral variables can have profound effects on health. The question of whether stress poses a risk for cancer progression has been difficult to answer. To conceptualize these relationships in adult humans, we proposed that stress accompanying a cancer diagnosis would trigger biologic responses, as well as psychological and behavioral responses, relevant to subsequent disease progression. A randomized clinical trial (RCT) was designed to test this possibility, reasoning that the receipt of a psychological intervention might serve as a protective mechanism to significantly alter the chain of adverse stress effects, and thereby, impact disease endpoints. Accruing breast cancer patients with Stage II or III disease (N = 227), earlier papers reported that patients randomized to the Psychological Intervention arm significantly improved across all secondary outcomes (psychological, health behaviors, adherence, health) as well as immunity (higher levels of PHA and ConA T cell blastogenesis) compared to patients randomized to the Assessment only arm. After a median follow-up of 11 years, we found a reduced risk for recurrence [Hazard Ratio (HR) = 0.55, P = .034] and improved survival (HR = 0.44, P = .016) among patients randomized to receive the psychological intervention.

For patients who recurred, we now test the hypothesis that Intervention patients (n=23), compared to the Assessment only patients (n = 18), would manifest more positive biobehavioral responses at recurrence and thereafter. Survival was also examined.

Methods. Psychological distress, quality of life, social support, health, relative dose intensity, and immunity [Natural killer cell cytotoxicity (NKCC); T cell proliferation in response to Concanavalin A (Con A) and phytohemagglutinin (PHA)] data were collected after recurrence diagnosis (baseline) and 4, 8, and 12 months later. Study arms were compared using mixed-effects or ANOVA models with two-sided tests.

Results. At recurrence diagnosis, comparable levels of baseline distress were found. However, significant improvements across all measures were found only for the Intervention arm, including depressive symptoms, social support, dose intensity, and immunity (NKCC and lymphocyte proliferation) (all Ps < .04). Cox analysis show that risk of death following recurrence was significantly higher in the Assessment arm (hazard ratio = 2.50; P = .010).

Conclusions. An effective psychological intervention, delivered when patients were initially diagnosed with breast cancer, provided enduring benefits when cancer returned. Novel data suggest that psychological interventions may achieve very long-term benefits, including improved survival, as patients continue the cancer trajectory.

Keywords: psychological, behavioral, immune

369 Center For Psycho-Oncology Research: A Mind Body Center

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We report on the results of 3 Projects that were conducted as part of a P50 Mind-Body Center grant (5 P50 CA84944) entitled "Center for Psycho-Oncology Research" conducted between 1999 and 2005. The focus of this Center was to test the effects of stress-related processes and stress management intervention on persons at risk for or being treated for different cancers. Three trials tested the effects of stress management interventions on quality of life indicators and a set of biobehavioral variables hypothesized to be related to health outcomes in persons at risk for cervical cancer (Project 1) and patients diagnosed with breast cancer (Project 2) and prostate cancer (Project 3). This research was coordinated through 4 Cores including an Administrative Core (Core A), a Psychosocial Assessment Core (Core B), a Biological Assessment Core (Core C), and a Data Management and Statistics Core (Core D). Approximately 500 patients participated in these trials.

Project 1 tested the effects of a 10-week group-based cognitive behavioral stress management (CBSM) intervention on quality of life (QOL) indicators and the development of cervical dysplasia in women at risk for cervical cancer based on co-infection with HIV and Human PapillomaVirus (HPV). This project found that HIV+HPV+ women assigned to CBSM showed decreases in perceived life stress and decreased prevalence and persistence of cervical neoplasia at 6 month follow-up. **Project 2** tested the effects of a 10-week group-based CBSM intervention on QOL indicators, positive and negative affect states, and endocrine and immune indicators in women who had completed medical treatment for early stage breast cancer at least 3 months prior to randomization. This project found that women assigned to CBSM showed decreases in negative affect (anxiety, depressed mood), increases in benefit finding and spirituality, decreases in urinary cortisol, and increases in natural killer cell cytotoxicity. **Project 3** tested the effects of a 10-week group-based CBSM intervention on QOL indicators, physical functioning, and biobehavioral variables in men being treated for early stage prostate cancer via either prostatectomy or radiation. This project found that men assigned to CBSM showed improvements in quality of life, increases in stress management skills, and benefit and improvements in urinary functioning. Secondary analyses revealed that CBSM was associated with improved sexual functioning in men who reported elevated distress at study entry. The Center was successful in establishing the efficacy of CBSM intervention for a diverse set of patients at risk for or diagnosed with relatively common cancers. There was consistent evidence that CBSM participants showed improvements in general QOL indicators as well as some indicators that were specific to the disease being studied. There was also consistent evidence that this intervention modulated objective indicators of stress such as the adrenal stress hormones. There was some evidence that CBSM was associated with improvements in indicators of immune functioning (natural killer cell activity) (Project 2) and reductions in disease promotion (cervical neoplasia) (Project 1). Further analyses of immunologic and disease activity indicators in some of the projects is ongoing. Longer-term follow-up of each of these cohorts is warranted in order to examine whether stress management intervention can affect quality of life and physical health over clinically meaningful periods.

Keywords: stress management, biobehavioral mechanisms, quality of life

370 Exercise for Health: Young Breast Cancer Survivors

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Background: Breast cancer is the most common form of cancer among US women in every major racial/ethnic group. In 2000, an estimated 26.4% of all cases were in women under age 50. Younger women are more likely to receive toxic multi-modal treatments that contribute to ovarian failure and early menopause, putting this growing group of women at risk for bone mineral loss, osteoporosis, and fractures. Reducing the risk is critical because young survivors have the greatest expected longevity.

Aims: This study aims to test the hypotheses that young breast cancer survivors randomly assigned to an exercise intervention, compared to controls, will after one year 1) perform resistance and aerobic exercise more frequently and for longer periods of time, 2) experience less bone loss and lower bone resorption as measured by DXA (dual energy X-ray absorptiometry) and biochemical markers of bone turnover, 3) be more likely to have a body mass index within recommended guidelines, 4) demonstrate a greater increase in lean body mass and a greater reduction in fat mass, 5) reduce risk factors for cardio-vascular disease and metabolic syndrome, and 6) report better mental and physical health.

Methods: In this trial, 400 women who were age 50 or under at diagnosis with invasive breast cancer and recent completion of chemotherapy are being randomized to the "Coach Approach" YMCA exercise program or the control group. Each woman in the exercise group receives a one-year YMCA membership and is assigned a Coach (personal trainer) who will assess her physical fitness, develop a tailored exercise program, monitor adherence, and provide social support and counseling. Women in the control group receive a monthly health newsletter. All women are being assessed at pre- and post-test (one year later) with bone density testing, laboratory evaluation, and questionnaires.

Significance: This research will advance scientific knowledge about the effects of abrupt menopause and weight gain due to adjuvant treatment on bone loss as well as cardiovascular, skeletal, and gonadal biomarkers that are not only prognostic risk factors for breast cancer, but also risk factors for chronic disease in young breast cancer survivors. Further this trial will examine the extent to which enrollment in an individualized YMCA exercise program emphasizing resistance training and cardiovascular conditioning reduces bone loss and risk factors for chronic disease in this population. The use of physiological and biochemical markers to measure bone turnover and risk factors and partnering with the YMCA to provide individually tailored exercise are innovations with this group of women. Program dissemination will be facilitated by the stable nationwide infrastructure of community-based Y facilities.

Keywords: exercise, resistance training, cardiovascular conditioning

371 Mindfulness-Based Intervention to Enhance Cell-Mediated Immune Response to HPV Among Women at Risk for Cervical Cancer

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Human papillomavirus (HPV) infection is a primary risk factor for cervical cancer. However, HPV infection does not lead to cancer in all cases. Data suggest that immune factors play a central role in controlling HPV infection and cervical disease progression. The relation between immune factors and cervical disease is particularly relevant given the following set of observations: 1) Receipt of an abnormal Pap test result is a stressful event that can lead to increased distress; 2) Psychological responses to stress are associated with immune dysregulation; and 3) Higher levels of stress have been associated with cervical disease progression. Over the past several years, we have been conducting a series of studies to evaluate psychosocial and behavioral correlates of lymphoproliferative responses to HPV16 among women with cervical dysplasia. Our findings suggest that higher levels of perceived stress are associated with impaired T-cell response to HPV16 (OR = 0.91, 95% CI = 0.85-0.98), suggesting a potential mechanism by which stress may influence cervical disease progression.

Based on these findings, we are currently conducting a randomized trial of a mindfulness-based stress reduction (MBSR) program among women with cervical dysplasia. MBSR is a standardized, eight-week group intervention that teaches self-regulation of attention through a set of formal and informal mindfulness practices. Our preliminary data indicated that MBSR contributes to significant improvements in quality of life and reductions in distress, anxiety, and medical symptoms ($ps < 0.01$) in a heterogeneous patient population. Importantly, participation in MBSR also resulted in significant alterations in various immune parameters, including enhanced natural killer cell functional activity and reductions in inflammatory cytokines ($ps < 0.05$). Thus, a primary aim of the present study is to examine the effects of MBSR on HPV-specific immune functioning (e.g., T-cell response to HPV16 E6, E7, and L1 peptides, intracellular cytokine expression in HPV-stimulated T cells) compared to a control condition at post-intervention and follow-up time points.

In summary, our findings suggest that psychosocial stress is associated with deficits in HPV-specific immune response. Given that participation in MBSR has been observed to lead to enhanced psychosocial and immunologic functioning, we are currently evaluating the effects of MBSR on cell-mediated immunity to HPV. These findings will contribute to a greater understanding of how alterations in biobehavioral pathways may influence susceptibility to cervical cancer in an at-risk population.

Keywords: behavioral intervention, human papillomavirus (HPV), cervical dysplasia

372 Do Cytokines TGF-α and Neuregulin-1 Suppress Circadian Clock Output in Mice? A Model for Cancer-Related Fatigue

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Circadian or daily rhythms modulate physiological responses. Robust daily rhythms are predictive of improved prognosis for cancer patients, independent of performance status measures. Disruption of circadian rhythms is associated with poor sleep quality and negative mood, fatigue, and reduced quality of life. The ability to care for a patient at home is often lost when the patient no longer sleeps during the night. The mechanism by which tumors suppress circadian rhythms and impair quality of life is unknown. We hypothesize that cytokine release induced by tumors may act directly in the neural system driving circadian rhythms and this action may induce fatigue and circadian rhythm disruption.

Previous studies have shown that central administration of the cytokines TGF-α and neuregulin-1 and systemic administration of IFN-α can disrupt behavioral rhythms in hamsters and mice, likely through action directly on suprachiasmatic nuclei neurons or on the direct targets of these neurons. As an animal model of tumor-induced disruption, we tested if peripheral administration of TGF-α and neuregulin-1 can similarly disrupt locomotor activity rhythms. Our initial experiments used systemic injections to administer the cytokines, and these were followed by more extensive studies using chronic infusion of NRG-1 with osmotic minipumps. Behavioral rhythms were measured both with general activity monitors and with running wheels. Mice treated with systemic injections of either TGF-α or NRG-1 showed dose-dependent suppression of wheel-running activity. Hamsters receiving 5 day infusions of NRG-1 showed decreased activity during the infusion period. This effect was limited to the more intense wheel-running activity; no significant changes in general cage activity recorded by the motion sensors were observed.

This study has demonstrated that systemic cytokines NRG-1 and TGF-α decrease wheel-running activity and lead to less robust locomotor rhythms. These data imply that when released in excess by tumors, these cytokines may also affect circadian rhythms in humans. This research will increase our understanding of the biological mechanism by which tumor growth can impact the circadian system. Effects on circadian rhythms can impact quality of sleep and levels of cancer-related fatigue, and might also affect progression of tumor growth.

Keywords: cytokine, neuregulin, circadian

373 Stress-Related Regional Brain Activation During A Verbal Declarative Memory Task In Metastatic Breast Cancer

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Women with breast cancer may have increased risk for cognitive impairments including memory and learning deficits. Given previous findings that diurnal cortisol levels tend to be flattened in women with breast cancer, abnormal stress response and hypothalamic-pituitary-adrenal (HPA) axis function may contribute to memory problems in these women.

We prospectively recruited 14 women with metastatic breast cancer and 14 age-matched healthy control females to undergo functional magnetic resonance imaging (fMRI) designed to measure functional brain activation associated with a verbal declarative memory encoding task. Baseline salivary cortisol was measured on two consecutive days at awakening, noon, 5pm and 9pm in both groups. Additional cortisol samples were obtained from women with breast cancer during the Trier Social Stress Task (TSST). Negative affect coping style was measured using the Weinberger Adjustment Inventory (WAI). Healthy women demonstrated significantly greater activation in the bilateral dorsolateral prefrontal cortex during the encoding task compared to women with breast cancer. Women with breast cancer demonstrated significantly greater activation in the left amygdala compared to healthy women. Additionally, women with breast cancer demonstrated significant deactivation deficits in the left hypothalamus meaning that they failed to appropriately inhibit hypothalamus activation during the encoding task. In women with breast cancer, left amygdala activation during the encoding task was significantly correlated with baseline cortisol slope ($r = .74$, $p = .001$), TSST cortisol ($r = .52$, $p = .01$) and WAI repressive/defensive score ($r = .38$, $p = .01$). Left amygdala activation also predicted memory recall accuracy ($F = 10.3$, $p = .009$) while hippocampus (and caudate, used as a control region) did not in women with breast cancer. In healthy control women, left hippocampus predicted recall accuracy ($F = 8.2$, $p = .002$). Cortisol collection/analysis is not yet complete for healthy controls.

Amygdala activation in women with breast cancer during the encoding task is surprising given that the task involved neutral stimuli and amygdala is typically involved in memory for emotional stimuli and activation of a stress response. Our findings suggest a stress-related sensitization of the amygdala such that seemingly innocuous stimuli are infused with emotional valence and thus recalled more intensely than is necessary. Perhaps cortisol-related amygdala sensitization is one mechanism underlying dysfunctional stress responses in chronically distressed individuals. Our results suggest that greater than expected amygdala activation could represent a neurobiological correlate of a less well-regulated physiological response associated with a repressive coping style. Additionally, prefrontal cortex deficits in women with breast cancer might also reflect a lack of top-down executive control over emotional responsiveness.

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Keywords: breast cancer, amygdala, cortisol

374 Neuroendocrine Modulation of STAT3 in Ovarian Cancer

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Objectives: Chronic stress is associated with cancer growth and progression, but the underlying mechanisms of this association are incompletely understood. We examined the role of STAT3, a transcription factor affecting many oncogenic pathways, in mediating invasion and metastasis pathways induced by the stress-associated hormones norepinephrine (NE), epinephrine (Epi), and isoproterenol (Iso).

Methods: In vitro, the epithelial ovarian cancer cell lines SKOV3 and EG were exposed to increasing concentrations of NE, Epi, or Iso, with and without beta-blockers (propanolol), alpha1- or alpha-2 blockers (prazosin or yohimbine). STAT3 activation, localization, and DNA binding were measured by Western blot, immunohistochemistry, and the Electrophoretic Mobility Shift Assay (EMSA). MMP-2 and MMP-9 levels were measured by ELISA. In vivo, orthotopic ovarian tumors were examined for patterns of metastasis (by inspection) and MMP-2 and MMP-9 expression (by immunohistochemistry) in mice subjected to chronic beta agonists, with and without liposomal STAT3-specific siRNA.

Results: NE, Epi and Iso induce phosphorylation of STAT3, with subsequent translocation to the nucleus and DNA binding, in a dose-dependent fashion in both SKOV3 and EG cell lines. STAT3 phosphorylation was inhibited by propanolol and by KT5720 (PKA inhibitor); but not by prazocin, yohimbime, or anti-IL-6 antibody. Cells stimulated with Iso had increased MMP-2 (by 2.4-fold) and MMP-9 (1.9-fold) production, but this increase was abrogated by STAT3 inhibition with anti-STAT3 siRNA. In mice stressed with daily Iso, tumors were significantly more invasive, and had increased MMP-2 and MMP-9 expression. However, downregulation of STAT3 with liposomal siRNA completely abrogated the increased invasiveness and MMP-2 and -9 induction by Iso.

Conclusions: Stress hormones acting through beta-adrenergic receptors induce STAT3 activation, with subsequent transport to the nucleus and DNA binding. Through STAT3, these hormones increase MMP-2 and MMP-9 expression, and tumor invasiveness. This pathway may play an important role in malignant progression, with particular importance in conditions of chronic stress.

Keywords: chronic stress, transcription factor, ovarian cancer

375 Biobehavioral-Cytokine Interactions in Ovarian Cancer

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Ovarian cancer is the second most common gynecologic cancer but has the highest mortality rates. Although relationships between biobehavioral factors and immunity in cancer have been well-documented, there has been little investigation of relationships between biobehavioral factors and molecules involved in angiogenesis and invasion. VEGF, a key promoter of tumor angiogenesis, is associated with poor ovarian cancer survival and is known to be stimulated by the stress hormones norepinephrine (NE) and cortisol. Matrix metalloproteineases (MMPs) -2 and -9 are critical to ovarian cancer invasion, and their production by ovarian tumor cells is also stimulated by NE. We hypothesized that patients with greater depressed mood and/or lower levels of social support would have higher levels of VEGF and greater production of MMPs-2 and -9 in the tumor microenvironment. We also examined genes that were differentially expressed in tumors from a subset of matched patients with high (high depression/low social support) and low levels of biobehavioral risk.

Patients suspected of ovarian cancer completed questionnaires within a week prior to surgery. Fifty-six patients were included in these analyses following histological confirmation of epithelial ovarian cancer. Tumor samples were analyzed for macrophage (CD68+) and tumor cell production of MMPs-2, -9, and vascular endothelial growth factor (VEGF) using confocal microscopy. *In vitro* stimulation of isolated macrophage cells by NE and cortisol was performed to assess effects on MMP-9. All analyses adjusted for cancer stage. Patients with higher levels of depressed mood ($p<0.0001$), current stress ($p=0.01$), life stress over the last 6 months ($p=0.004$), and general negative affect ($p=0.007$) demonstrated significantly greater MMP-9 in CD68+ cells. In contrast, higher social support was associated with lower tumor production of MMP-9 ($p=0.023$) and VEGF ($p=0.036$). *In vitro* analyses demonstrated directly enhancment (up to a 2-fold increase) of macrophage MMP-9 production by NE and cortisol. Subset analyses examining gene expression identified specific signal transduction pathways indicating altered gene expression profiles from tumors of patients with high vs. low biobehavioral risk.

These data demonstrate that biobehavioral factors are related to production of key factors involved in angiogenesis and invasion by tumor cells and by tumor associated macrophages. *In vitro* enhancement of stromal MMP-9 production by stress hormones was also demonstrated. Moreover, differential gene expression was inherent at the level of the tumor *in vivo*. These findings suggest that biobehavioral factors may directly influence angiogenesis, invasion, and transcriptional activities of tumor cells, and thus may influence tumor progression in ovarian cancer. Findings have implications for innovative behavioral and pharmacological intervention strategies for ovarian cancer patients.

Keywords: ovarian cancer, biobehavioral, angionenesis

376 PNI-Based Stress Management in Early Breast Cancer

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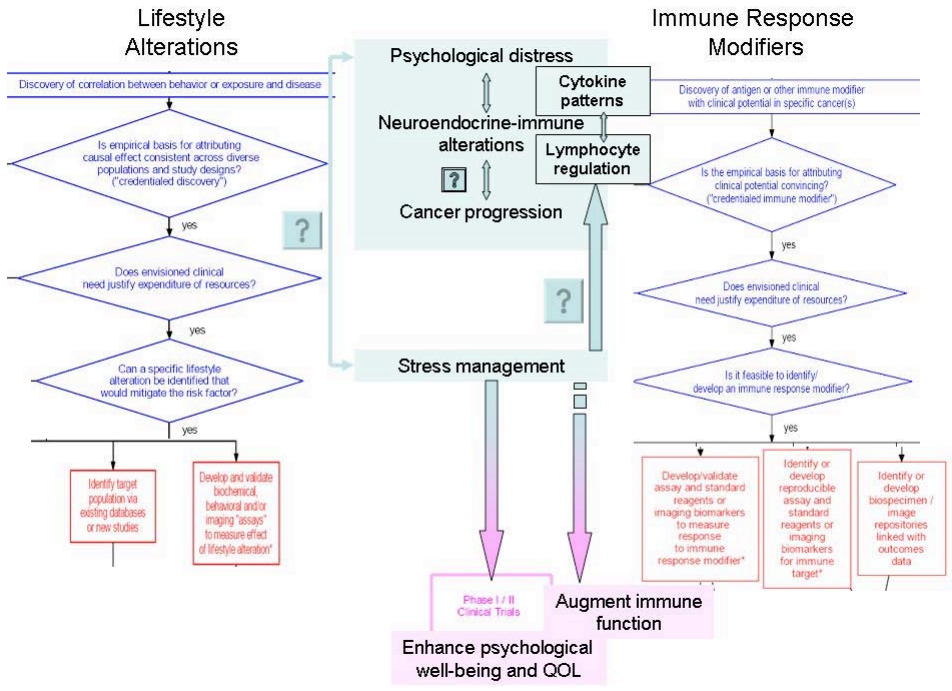
The overall purpose of this randomized clinical trial is to determine whether two mind-body-spirit approaches for stress management will enhance psychosocial functioning, quality of life, and physical health among women receiving adjuvant chemotherapy for early breast cancer (Stage I or II). Multiple indicators derived from the PNI paradigm are being measured within these three outcome domains, along with neuroendocrine mediation. The interventions, focused tai chi (TCHI) training and spiritual growth groups (SPRT), are designed to reduce perceived stress and enhance adaptational outcomes. Primary hypotheses are that the interventions will (a) enhance psychological well-being and quality of life (QOL) and (b) perhaps augment immune function. Thus, this study addresses two of the NCI pathways for early translational research: Lifestyle Alterations and Immune Response Modifiers.

AIM 1 is to compare the TCHI and SPRT interventions and a standard care control condition for effects on *psychosocial functioning* (perceived stress, coping patterns, spirituality, social support, inner strength, depressive symptoms, benefit finding), *quality of life*, *neuroendocrine mediation* (cortisol, beta-endorphin, leu-enkephalin), and *physical health* (immune status, symptom distress, and cancer-related health status, including fatigue). AIM 2 is to test the theoretical model by examining predicted relationships among the selected PNI-based indicators using structural equation modeling.



Within the Lifestyle Alterations pathway, our work is focused on the *credentialed discovery* trajectory.

Hypothesized effects of stress management include reduced psychological distress, thereby impacting PNI mechanisms that may affect cancer progression. Research is aimed at identifying effective biobehavioral interventions that may mitigate the psychological distress associated with treatment for breast cancer.

In the Immune Response pathway, our work may illuminate immunological mechanisms and thereby shed light on *supporting tools* that may arise from further understanding of PNI mechanisms, particularly changes in cytokine patterns and lymphocyte function.

Additional collaborative and multidisciplinary research may lead to Phase 1/II clinical trials aimed at enhancing immune function (e.g., administration of cytokines), which may slow or halt cancer progression by augmenting neuroendocrine and immune function), leading to reduced psychological distress.

Keywords: breast cancer, psychoneuroimmunology, stress management

377 Walking Forward: NIH Disparity Project to Lower Cancer Mortality Rates For American Indians in Western, South Dakota

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Purpose/Objective(s): American Indians (AIs) suffer higher cancer mortality rates compared to non-AIs. Under the Cancer Disparities Research Program of the NCI, we are researching methods to improve cancer treatment and outcomes for AIs in Western, SD.

Materials/Methods: This community based participatory research program consists of patient navigation, clinical trials, surveys to evaluate barriers to access, and a molecular study (ATM) as a potential means to assess radiation toxicities. The success of enrolling AIs on this program since September 2003 is detailed.

Results: As of July 2008, 283 have undergone patient navigation, 12 have participated in cooperative group clinical trials, 7 in CDRP investigator lead trials (IMRT and brachytherapy), 955 have taken the community survey, 56 the cancer survey, and 42 have undergone ATM testing. The clinical trial participation rate was 21%. Reasons for non-participation in clinical trials were assessed and will be presented. AI cancer patients undergoing PN during treatment had less treatment interruptions (3 days) and higher completion rates compared to non-navigated patients. The surveys revealed the following: AIs presented with significantly higher rates of advanced-stage cancer ($p=0.04$), scored lower on a cancer screening knowledge battery ($p=0.0001$), expressed more negative attitudes toward cancer treatment, expressed significantly higher levels of mistrust ($p=0.0001$) and lower levels of satisfaction with prior experience with health care ($p= 0.0001$) compared to non-AI patients. From 1,000 participants, the reported screening rates were 61% for breast cancer, 49% cervix, 24% colorectal females, 14% colorectal males, and 32% for prostate.

Conclusions: We have successfully enrolled members of an underserved population into various aspects of our program with a clinical trial participation rate of 21%, compared to < 1% nationally. We have identified barriers to cancer screening and treatment. Based upon results from the first 5 years, the next cycle of study will expand patient education, screening, navigation, clinical trials, genomics, and palliation.

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Keywords: disparities, American Indians, radiation oncology

378 Blockade of the Renin-Angiotensin System Leads to Prevention or Amelioration of Radiation-induced Cognitive Impairment

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Progressive cognitive impairment occurs in up to 50% of brain tumor patients surviving ≥ 6 months after receiving fractionated partial or whole-brain irradiation (WBI). This is of major concern for the 200,000 patients who present with primary and metastatic brain tumors/year. There are currently no long-term treatments or preventive approaches for radiation-induced cognitive impairment. Experimental studies have demonstrated clearly that radiation-induced late effects are amenable to treatment. One of the most effective approaches has been blockade of the renin-angiotensin system (RAS). Angiotensin-converting enzyme inhibitors (ACEI) or angiotensin type 1 receptor antagonists (AT_1RA) have proved highly effective in the treatment and prevention of radiation-induced late effects in the kidney and lung, independent of any reduction in blood pressure. We hypothesized that RAS blockade also prevents and/or ameliorates radiation-induced cognitive impairment.

We used a well-characterized rat model in which fractionated whole-brain irradiation (40 Gy, 8 fractions of 5 Gy, twice/week for 4 weeks) of the young adult male rat leads to a chronic, progressive reduction in cognitive function that is statistically significant at 26 and 52 weeks post-irradiation. Groups of young adult (12-14 week old) male Fischer 344xBrown Norway rats received either: i] fractionated WBI, ii] sham-irradiation; iii] fractionated WBI plus the AT_1RA, L-158,809 (Merck & Co., Inc; 20 mg/L drinking water), and iv] sham-irradiation plus L-158,809. Rats received the L-158,809 three days prior to the start of WBI and were maintained on the AT_1RA during and after fractionated WBI for up to 54 weeks post-irradiation. Cognitive function was assessed using the novel object recognition test.

Administration of L-158,809 prior to, during, and for 28 or 54 weeks after fractionated WBI prevented or ameliorated the radiation-induced cognitive impairment observed 26 and 52 weeks post-irradiation. These radiation-induced cognitive impairments occurred without any gross histological changes. Moreover, giving L-158,809 prior to, during, and for only 5 weeks post-irradiation ameliorated the significant cognitive impairment observed 26 weeks post-irradiation. These AT_1RA are: i] routinely prescribed for the treatment of hypertension, ii] well-tolerated, and iii] exhibit antitumor effects. Thus, they appear to be ideal drugs for future clinical trials, offering the promise of improving the quality of life for brain tumor patients who receive fractionated WBI.

References: Brown WR et al. Radiat Res 164:662, 2005. Atwood T et al. Radiat Res 168:574, 2007.

Keywords: fractionated whole-brain irradiation, cognitive impairment, AT_1 receptor antagonists

379 Palifermin Reduces the Incidence of Chemoradiotherapy-Induced Severe Oral Mucositis in Patients With Head and Neck Cancer

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Keratinocyte growth factor (KGF) has remarkable cytoprotective effects, particularly in the gastrointestinal tract. A series of preclinical, translational and clinical studies culminated in regulatory approval for the use of palifermin (a truncated KGF derivative) to reduce the incidence and duration of severe oral mucositis (OM) in patients with hematologic malignancies who receive high dose chemoradiotherapy (CT/RT) prior to peripheral blood progenitor cell transplantation.

Recently, two phase III multinational clinical trials were completed to test the safety and efficacy of palifermin in patients with locally advanced head and neck cancer (HNC). One of the studies was a randomized, double-blind, placebo-controlled trial in which 188 adults were enrolled who had newly diagnosed inoperable stage III/IVA,B squamous cell HNC. Patients received CT/RT (conventionally fractionated RT 2 Gy/day to 70 Gy and concurrent cisplatin 100 mg/m^2 on days 1, 22 and 43) and were randomized 1:1 to receive IV palifermin (180 mcg/kg) or placebo 3 days before the start of CT/RT, and once weekly during a 7-week CT/RT course. The incidence of severe OM (primary endpoint) was significantly reduced in the palifermin subjects compared with placebo subjects (54% vs. 69%; p=0.041). Overall survival (median follow-up of 48 weeks) was compared using Kaplan-Meier curves and the stratified log-rank-test, and no difference between palifermin and placebo subjects was observed (p=0.721).

The other study was a randomized, double-blind, placebo-controlled trial in which 186 adults were enrolled with resected stage II-IVB squamous cell HNC and were treated with adjuvant CT/RT (conventionally fractionated RT 2 Gy/day to 60 Gy and cisplatin 100 mg/m^2 on days 1, 22 and optionally on day 43). Patients were randomized 1:1 to receive IV palifermin (120 mcg/kg) or placebo 3 days before the start of CT/RT and once weekly during the 6-week CT/RT course. The incidence of severe OM (primary endpoint) was significantly reduced in the palifermin subjects compared with the placebo subjects (51% vs. 67%; p=0.027). Overall survival (median follow-up 63 weeks) was compared using Kaplan-Meier curves and the stratified log-rank-test, and no difference in subjects was observed (p=0.83).

In conclusion, palifermin was safe and significantly reduced the incidence of severe OM in patients with locally advanced inoperable HNC undergoing CT/RT and in patients with resected locally advanced HNC undergoing adjuvant CT/RT.

References: Spielberger R et al. N Engl J Med 351: 2590, 2004. Finch PW and Rubin JS JNCI 98: 812, 2006.

Keywords: palifermin, oral mucositis, head and neck cancer

380 Mechanisms of Chemotherapy-Induced Cognitive Changes: Prospective Structural, Functional, and Diffusion Tensor Brain MRI in Breast Cancer Patients

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Although follow-up and prospective studies of cancer survivors have demonstrated adverse cognitive changes in some patients after systemic chemotherapy, the neural substrate of these changes remains unclear. We and others have proposed a range of mechanisms that might account for cognitive changes (e.g., neurotoxicity, neurodegeneration, microvascular changes, inflammation, neural transmission, etc.). Advanced brain imaging techniques have the potential to elucidate these mechanisms. In a prospective study using 1.5T MRI before and after chemotherapy, we employed voxel-based morphometry (VBM) and diffusion tensor imaging (DTI) to examine changes in gray and white matter (GM, WM) and fMRI during an auditory verbal n-back working memory task to assess changes in brain activity. Participants included healthy controls (HC) and two groups of breast cancer patients. The CTx+ group received systemic chemotherapy in addition to surgery, local radiation, and/or hormone therapy; the CTx- group did not receive chemotherapy. Groups were matched for age, gender (all women), and education. High resolution T1-weighted MRI volumes and DTI were acquired as part of a comprehensive protocol at baseline (BL; after surgery but before radiation and/or chemotherapy) and one month (M1) and one year (Y1) following the completion of chemotherapy, or yoked intervals for the CTx- and HC groups. We predicted that changes on imaging would be detectable at M1 followed by partial recovery at Y1. At M1 relative to BL the CTx+ group showed significantly decreased GM density in bilateral frontal, thalamic and hippocampal regions on VBM and increased trace diffusivity on DTI in distributed central WM regions bilaterally. These changes were not seen in the HC or CTx- groups. In parallel, decreased activation in frontal regions was observed on fMRI during working memory performance. As predicted, there was significant partial recovery from these initial post treatment changes.

Overall, project findings to date suggest decreases in cerebral GM density and WM integrity as well as altered brain activation shortly after systemic chemotherapy with partial recovery on one year follow-up. Future directions: A new cohort is presently being studied using 3.0T MRI with the addition of arterial spin labeling (ASL) perfusion measurement and spectroscopic imaging (MRSI) to further clarify the basis of cognitive changes. Analysis of candidate gene pathways and plasma biomarkers are also ongoing. Advanced neuroimaging techniques can help clarify the neural basis of changes after systemic cancer chemotherapy.

Keywords: chemotherapy, neuroimaging, cognition

381 Activating Endogenous Mechanisms That Enhance Anti-Tumor Immune Responses

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Fighting disease by harnessing endogenous, natural, defense mechanisms in conjunction with pharmacological and surgical interventions is likely to be clinically beneficial. The fight-or-flight stress response is one of nature's under-appreciated defense mechanisms that activates multiple psycho-physiological systems to promote survival. We have proposed that it may be useful to identify biological mechanisms by which a fight-or-flight stress response promotes survival because these mechanisms could be clinically harnessed to fight disease. Stress can be defined as a constellation of events that begins with a stimulus (stressor), that precipitates a reaction in the brain (stress perception), that activates physiologic fight/flight systems in the body (stress response). The idea that a stress response may be harnessed to fight disease seems counter-intuitive at first glance because numerous studies have shown that chronic stress/distress can suppress or dysregulate immune function (Nat Rev Immunol, 2005). For example, studies conducted in our laboratory have shown that chronic stress suppresses cell-mediated immunity, enhances regulatory T cell numbers, and increases susceptibility to ultraviolet-B (UVB)-induced squamous cell carcinoma (SCC) (Brain, Behavior, Immunity 1997; J Nat Cancer Inst, 2005).

However, in contrast to chronic (weeks to months) stress, an acute (minutes to hours) fight-or-flight stress response is one of nature's fundamental psycho-physiological survival mechanisms (e.g. without this response, a lion has little chance of catching a gazelle, and a gazelle has little chance of escape). We hypothesized just as the acute stress response prepares the brain, heart, and muscles for fight or flight, it may also prepare the immune system for challenges (e.g. wounding or infection) that may be imposed by a stressor (e.g. predator). This hypothesis was confirmed by studies showing that acute stress experienced at the time of immune activation or antigen exposure induces a redistribution of circulating immune cells, increases leukocyte trafficking to sites of wounding or immune activation, enhances innate and adaptive immunity, and induces a long-lasting enhancement of cell-mediated immunity (J. Immunol 1995, 1996; PNAS, 2000, 2005; Amer J Physiol, 2005; Int Immunol, 2005). Based on these studies, we set out to examine the effects of acute stress-induced immuno-enhancement in the context of cancer. Because SCC is an immuno-responsive cancer, we hypothesized that acute stress experienced immediately prior to UVB exposure, would enhance cell-mediated immunity and increase resistance to SCC. To test this hypothesis, mice were exposed to UVB (minimum erythemal dose, 3-times/week, weeks 1-10). The control and acute stress groups were treated identically except that the stress group was restrained for 2.5 h before each of nine UV sessions during weeks 4 to 6. Tumors were measured weekly, and tissue collected at weeks 7, 20, & 32. Chemokine and cytokine gene expression was measured by quantitative PCR, and CD4+ and CD8+ T cells were enumerated by immunohistochemistry and flow cytometry. Mice that were acutely stressed showed greater cutaneous T cell attracting chemokine (CTACK)/CCL27, RANTES, IL-12, and IFN-γ gene expression (at weeks 7, 20, & 32), higher T cell numbers in skin (weeks 7 & 20) and blood (week 20), and fewer tumors (weeks 12 to 19).

These results suggest that activation of acute stress physiology increases leukocyte trafficking and/or function during/following UV exposure, and produces a long-lasting enhancement of Type 1 cytokine-driven cell-mediated immunity that is crucial for resistance to SCC. These findings begin to identify psycho-physiological mechanisms that may be harnessed through pharmacological and/or bio-behavioral interventions to enhance immune system mediated elimination of tumors that are either naturally immunogenic, or rendered immuno-responsive via tumor immunotherapy.

Keywords: adjuvant immune-enhancement, psychological stress, skin cancer, squamous cell carcinoma

382 Biology and Prognostic Significance of FLT3 Mutations in AML

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FLT3 encodes a receptor tyrosine kinase that regulates hematopoietic stem cell differentiation and proliferation. Activating mutations of FLT3 can occur as a result of an internal tandem duplication of the juxtamembrane domain (FLT3 internal tandem duplication, FLT3/ITD) or a missense mutation of the activation loop domain (FLT3 activation loop mutation, FLT3/ALM). FLT3/ITD occurs in acute myeloid leukemia (AML) with an age-dependent increase in prevalence from <1% in infant AML to >40% in those older than 60 years, and its presence is associated with high relapse risk and poor outcome. Incidence of FLT3/ALM remains constant in (5-7%) in all age categories, and its presence does not have prognostic significance. Although FLT3/ITD portends higher relapse rate, 25-30% of those with FLT3/ITD do not relapse. We have demonstrated that structural characteristics of FLT3/ITD may affect its biology and clinical significance. Allelic variation of FLT3/ITD, which is a measure of mutant to wild type allele, accurately defines relapse risk, where those with FLT3/ITD allelic ratio (ITD-AR) of greater than 0.4 are at extremely high risk of relapse (>90% RR), whereas those with lower allelic ratio have a RR similar to FLT3 wild type (FLT3/WT) patients. Allelic ratio determination of FLT3/ITD as means of risk-status determination has been incorporated into the current phase III COG AML trial, where those with high ITD-AR are allocated to receive allogeneic stem cell transplant in first CR. Underlying mechanism for allelic ratio variation was studied using SNP/CGH array, demonstrating that copy-neutral LOH (CN-LOH) mediates the allelic variation. Expression profiling in those with CN-LOH identified dys-regulation of genes involved in homologous recombination and DNA segregation pathway. Specific genes are under evaluation for their role in mediating CN-LOH, which may be related to disease resistance.

Structural analysis of FLT3/ITD revealed that in all cases of ITD, amino acid residues Y591-Y597 were duplicated. This region, which encodes the switch and zipper regions of the juxtamembrane (JM) domain of FLT3, plays an important role in directing an optimal orientation of the autophosphorylation 'switch' residues and maintaining the autoinhibited conformation. JM-ITD insertions are expected to disrupt the autoinhibited conformation of the JM switch (JM-S) region, thus preventing proper kinase inhibition. In addition, length and the region of the mutation impacts clinical outcome, where those with longer ITD or those ITDs that involve the STAT5 binding domain (Y589/591) have a higher incidence of relapse and worse outcome.

Evaluation of FLT3 expression level in patients with FLT3/WT demonstrated significant variation in FLT3 expression where nearly $1/4^{th}$ of the patients had 20-100 fold higher FLT3 expression compared to the normal marrow controls. High FLT3/WT expression was associated with higher relapse risk. Leukemic blasts with high FLT3/WT expression had a higher response rate to FLT3 inhibitor Lestarutinib, suggesting that patients with high FLT3/WT expression may respond to FLT3 inhibitors. We demonstrate a level of complexity in FLT3 structure and function not previously appreciated. Complete understanding of the underlying mechanism and biology for FLT3 structural and expression variation would enable more appropriate therapeutic intervention.

Keywords: FLT3, internal tandem duplication, acute myeloid leukemia

383 Oncogenomics to Target the Myeloma Cell in its Bone Marrow Microenvironment

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Advances in oncogenomics and increased understanding of the role of the bone marrow (BM) in the pathogenesis of MM have provided the framework for a new treatment paradigm targeting the tumor cell and its BM microenvironment to overcome drug resistance and improve patient outcome. Immunomodulatory drugs thalidomide and lenalidomide, as well as proteasome inhibitor Bortezomib, are such agents which have rapidly translated from the bench to the bedside and transformed treatment of patients with MM. Ongoing efforts are identifying next generation therapies in MM on the one hand, and using oncogenomics to inform the design of combination trials on the other. Examples of promising novel targeted therapies include agents targeting the tumor cell surface (CD40, CS-1), cytokines (VEGF, BAFF), and intracellular targets (MEK, PI3K/Akt, NF-κB, cyclin D, proteasomes). We have gone on to use oncogenomics to define combination therapies to enhance cytotoxicity and overcome drug resistance against MM cells in the BM milieu. Based upon preclinical studies, Bortezomib has been combined with hsp 90 inhibitors, DNA damaging agents, Akt inhibitors, immunomodulatory drugs, and histone deacetylase inhibitors; and lenalidomide has been combined with steroids, proteasome inhibitors, and humanized monoclonal antibodies, and all combinations have already demonstrated clinical promise. This new paradigm for overcoming drug resistance and improving patient outcome in MM therefore has great promise not only to change the natural history of MM, but also to serve as a model for targeted therapeutics directed to improve outcome of patients with other hematologic malignancies and solid tumors as well.

Reference: Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC: Understanding multiple myeloma pathogenesis and the role of bone marrow microenvironment to identify new therapeutic targets. Nat Rev Cancer 2007; 7: 585-98.

Keywords: multiple myeloma, targeted therapy, microenvironment

384 Strategies for Overcoming Apoptosis and Microenvironment-Mediated Resistance in Acute Myelogenous Leukemia

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Resistance to chemotherapy following initially successful induction chemotherapy has been the major conundrum of leukemia therapy for the last 50 years. This programs focus is the identification of factors making AML cells and stem cells resistant to chemotherapy, identifying new therapeutic targets and conducting clinical trials with targeted therapeutics. The program has recently reported that mice with nur77 and nor1

knockout develop AML and that these nuclear receptors are absent in AML versus normal stem cells. Treatment in vitro with HDAC inhibitors re-establishes their expression. Nur77 has been associated with the conversion of Bcl-2 from an anti- into a pro-apoptotic protein. Bcl-2 family members were shown to be critical and are targeted by BH-3 mimetics including Obatoclax and ABT-737/263., which are now in clinical trials. In pre-clinical studies, ABT-737 was highly effective against AML and AML stem cells and was synergistic with MAPK inhibitors , in large part because of the additional inhibition of MCL-1. Other targets in the apoptotic pathway include XIAP and Survivin, for which pre-clinical rationale was developed and clinical studies are ongoing using antisense oligonucleotides. Small molecule inhibitors are under development under the auspices of this grant. A novel mechanism for the inactivation of p53 has been identified, (MDM2/HDM2), which is being targeted by small molecule inhibitors (Nutlin) . A phase I clinical trial is in progress, aiming at the disruption of HDM2/p53 protein/protein interactions. A completely novel level of gene regulation has become apparent with the discovery of microRNAs (miRs), including those regulating Bcl-2 and MCL-1 (miRs 15,16,29b). Our group reported a very large survey of all miRs in AML and was able to identify prognostic subgroups not recognized by cytogenetics. Functional studies are underway targeting specific miRs with the goals of restoring apoptosis signaling. Data are being correlated with proteomics data (presently >150 proteins and phosphoproteins) derived from >650 patients with AML by Reverse-Phase Protein Arrays (RPPA), and with our published analysis of all HOX genes in AML.. The stem cell transplant component of our program has developed I.V. Busulfan into the standard of care with FDA approval and fludarabine/busulfan as a highly effective regimen for AML. Non-myeloablative SCT was developed as effective therapy in particular for elderly patients in CR1.

Our group was first to identify the bone marrow microenvironment as a resistance factor in AML and developed strategies to disrupt leukemia/stroma interactions in vitro and in in vivo models. These basic studies have resulted in two protocols combining CXCR4 inhibition utilizing AMD3100 (Plerixafor) with the fludarabine/busulfan SCT regimen, and with Sorafenib, which we identified as an excellent FLT-3 inhibitor.

Overall, the program has successfully identified new therapeutic targets in AML, related to impaired apoptosis signaling and to the micro-environment which protects AML stem cells .

Keywords: apoptosis, tumor microenvironment, leukemia

385 Total Therapy (TT) for Myeloma (MM): 10% Cure Rate With TT1 Suggested by >10Yr Continuous Complete Remission (CCR); Bortezomib in TT3 Overcomes Poor-Risk Associated with T(4;14) and DelTP53 in TT2

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Background: The introduction of transplants in the 1980's and novel agents in the 1990's has markedly improved MM survival. TT trials applied all active treatment ingredients up-front with the objective to maximize long-term disease control. As novel agent combinations are increasingly being applied at the exclusion of transplants, it appears useful to establish a historical framework of long-term outcomes with our TT approach introduced in 1989.

Methods: An update is provided of overall survival (OS), event-free survival (EFS), CR rates and CR durations for TT1 (n=231; phase II, interferon maintenance), TT2 (n=668; phase III, +/- thalidomide, post-transplant consolidation) and TT3 (n=303; phase II, added bortezomib and thalidomide throughout).

Results: Stringently defined CR increased significantly from 40% in TT1 to 50% in TT2 to 60% in TT3. Median CR duration increased from 2.5yr in TT1 (16 in CCR beyond 10yr) to 5.0yr in TT2; the 3-yr estimate in TT3 is 90%. Median EFS and OS were 2.6yr and 5.7yr for TT1 and 5.0yr and 9.0yr for TT2; the 3-yr estimates in TT3 are 80% and 85%. A gene array-based high-risk score adversely affected OS, EFS and CR duration in both TT2 and TT3. However, the independent adverse implications, for all 3 endpoints, of t (4;14) and TP53 deletion observed in TT2 did not pertain to TT3, supporting a major role of bortezomib, added in TT3, for the management of these hitherto high-risk MM subsets.

Conclusions: A CCR plateau apparent at 10yr in TT1 is consistent with a ~10% cure rate. Significant improvements beyond TT1 with TT2 and especially TT3 bode well for marked increases in 10-yr OS and cure rates. Knowledge of t(4;14) and delTP53 status is key to identifying MM subsets uniquely benefiting from bortezomib.

Keywords: myeloma, molecular genetics, cure

386 Antibody-Targeted Therapeutics for B-cell Malignancies: From Bench to Clinic

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A variety of antibody-based therapeutics have been developed, with the greatest clinical impact being in the treatment of hematopoietic tumors, particularly B-cell types. We have focused on developing humanized monoclonal antibodies (MAbs) for use alone or as conjugates with radionuclides, cytokines or drugs, such against as CD20, CD22, CD74, HLA-DR, MUC1, and CEACAM6.

Our goal is to examine how these antibodies or antibody-conjugates can be best used to treat non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM), proceeding from cell culture, xenografts models, Cynomolgus monkeys, to patients, collaborating with 2 biotechnology firms and involving 4 academic research institutions, and involving discovery, preclinical testing, antibody engineering, GMP manufacturing, and Phase I/II clinical trials. Over the past 3 years of PO1 funding from the NCI for preclinical development, we have advanced 2 novel therapeutic agents to Phase-I or Phase- I/II clinical testing (veltuzumab, humanized anti-CD20 IgG in NHL), milatuzumab (humanized anti-CD74 in NHL, MM and CLL), as well as developing a novel CD20-bispecific antibody system for pretargeting radionuclides for both imaging (ImmunoSPECT and ImmunoPET) and therapeutic uses. This latter system is currently under the initial stages of product development and manufacturing for introduction into the clinic in the future, following encouraging preclinical results (Sharkey et al., Cancer Res. 2008 Jul 1;68(13):5282-90), especially in combination with anti-CD20 MAb (veltuzumab) immunotherapy, based on preclinical findings showing an efficacy advantage for this combination (Mattes et al., Clin Cancer Res, in press).

Encouraging preclinical results with an anti-CD20 MAb-intereron-□2b conjugate also justifies translating these findings to patients. The Dock-and-Lock (DNL) technology being developed for constructing multivalent, multifunctional antibodies is also being applied to the development of pretargeted immunoSPECT and immunoPET with various candidate targets of NHL and MM in order to improve specific targeting and imaging, to be compared to FDG-PET in these indications, especially for assessing therapeutic response and minimal residual disease. Other projects include DNL-engineered hexavalent anti-CD20 and anti-CD20/CD22 bifunctional constructs, studying both mechanisms of action in comparison to parental bivalent MAbs as well as their candidacy for clinical trials. Thus, these projects combine several unique antibody constructs in a multidisciplinary, highly translational, well-integrated, program that rapidly transfers new agents from the laboratory to the clinic with the goal of improving the therapeutic prospects in 3 B-cell malignancies. (Supported in part by NCI grants P01-CA103985 and R21-CA126060 from the National Institutes of Health.)

Keywords: non-Hodgkins lymphoma, immunotherapy, radioimmunotherapy

387 Potentiation of Histone Deacetylase Inhibitor Activity in Hematopoietic Malignancies by Agents That Interrupt the NF-κB Pathway

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Histone deacetylase inhibitors (HDACIs) are epigenetically-acting agents that have recently been approved for the treatment of cutaneous T cell lymphoma, and are currently under active investigation in patients with hematopoietic and other malignancies. Previous work from our laboratory and others has shown that in transformed cells, HDACIs induce acetylation of p65/RelA, thereby promoting NF-κB activation, which leads in turn to transcription of various pro-survival NF-κB-dependent genes, including $MnSOD_2$, Bcl-x_L, and XIAP, among others. Conversely, interruption of NF-κB signaling opposes HDACI-mediated p65/RelA acetylation/activation, thereby blocking upregulation of these anti-apoptotic proteins and promoting cell death via a JNK-dependent process (Dai et al., Mol Cell Biol 25:5429-44, 2005). We have also found that HDACIs interact synergistically with certain cytotoxic agents (e.g., fludarabine) in malignant hematopoietic cells through multiple mechanisms, including induction of oxidative damage, modulation of NF-κB activity, downregulation of DNA repair proteins, and potentiation of DNA damage. Based on these findings, a strategy has been developed designed to enhance the activity of HDACIs in hematopoietic malignancies by interrupting compensatory cytoprotective signaling pathways. One such approach is based upon the ability of the CDK inhibitor flavopiridol to inhibit a) IKK, and by blocking phosphorylation/degradation of IκBα, to prevent NF-κB activation; and b) the cyclinT/CDK9 pTEFb transcription complex, thereby antagonizing HDACI-mediated transcription of $p21^{CIP1}$ and other short-lived anti-apoptotic proteins such as XIAP. Preclinical studies demonstrated a high degree of synergism between the pan-HDACI vorinostat and flavopiridol *in vitro* and *in vivo*. These observations prompted the initiation of a multi-institutional Phase I trial of vorinostat and flavopiridol, along with correlative laboratory studies, in patients with refractory AML/MDS, which is currently ongoing. A second strategy involves the use of proteasome inhibitors (e.g., bortezomib) which prevent NF-κB activation directly by blocking the degradation of IκBα. Synergistic preclinical interactions between vorinostat and bortezomib have been observed in malignant lymphoid malignancies (e.g., DLBCL) and, more recently, bortezomib has been shown to interact in a highly synergistic manner with depsipeptide (romidepsin) in CLL cells in association with NF-κB inactivation (Dai et al., Clin Cancer Res 14:549-58, 2008). These preclinical findings have prompted the development of a multi-institutional Phase II trial of vorinostat and bortezomib in patients with refractory DLBCL and mantle cell lymphoma which has recently begun, and plans for a Phase I trial of romidepsin and bortezomib in patients with refractory CLL are underway. In summary, these initiatives should permit testing of the hypothesis that preclinical evidence of promotion of HDACI lethality by NF-κB interruption can be translated into improved activity of HDACIs in patients with various hematologic malignancies.

Keywords: HDAC inhibitor, NF-kB, apoptosis

388 The Myeloproliferative Disorders Research Consortium (MPD-RC)

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The Myeloproliferative Disorders Research Consortium (MPD-RC) focuses on the Philadelphia (Ph) chromosome negative MPD including polycythemia vera (PV), and idiopathic myelofibrosis (IM). The goals for the MPD-RC include: 1) Establishment of a multi-institutional international research group entitled the "MPD Research Consortium" which coordinates basic and clinical research dealing with the cellular and genetic foundations of the Ph negative MPD. 2) Establishment of a multi-institutional MPD Clinical Consortium which enables uniform, high volume sample collection, storage and distribution. 3) Performance of rationally designed clinical trials in patients with Ph negative MPD at multiple institutions. 4) Maintenance of an interactive website for MPD Consortium investigators, a sophisticated international tissue bank and an on-line database which allows for integration of basic and clinical research. This unique interactive relationship between talented basic researchers and clinical scientists has permitted the MPD Research Consortium to develop novel clinical treatment programs for Ph negative MPD and to identify specific biomarkers that are useful as indicators of therapeutic response and/or risk reduction. The program has six major projects: Project 1: Genetic Basis of Polycythemia Vera; Project 2: Mechanisms and Effects of NF-E2 and PRV-1 Overexpression in PV: Role of Jak2V617F; Project 3: Animal Models of Polycythemia Vera; Project 4: Mouse Models of Myelofibrosis; Project 5: Abnormal Stem Cell Trafficking in Myelofibrosis; Project 6: MPD Clinical Consortium which pursues clinical trials in PV and IM. The six projects are supported by three cores: Core A: Administrative Core; Core B: Biostatistics and Data Management; and Core C: Tissue Bank. These unique interactions between clinical and laboratory investigators which are interwoven within the MPD Research Consortium have lead to an improved understanding of the patho-biology of the Ph negative MPD as well improved strategies which assist in the diagnosis and treatment of these disorders.

Keywords: Philadelphia (Ph) chromosome negative, polycythemia vera (PV), idiopathic myelofibrosis (IM)

389 Molecular Targeting of Mitochondrial Hsp90 Chaperones for Novel Cancer Therapeutics

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Despite an unprecedented understanding of cancer genes and their pathways, the mortality rates of most cancers have changed little in thirty years, and mainstay cancer therapy has reached a plateau in the treatment of many tumors. Although new agents are urgently needed, a formidable hurdle in cancer drug discovery is the extraordinary molecular and genetic heterogeneity of human tumors. By the time of detection, most epithelial malignancies carry hundreds of mutated, deregulated or abnormally expressed gene pathways, making it unlikely to identify a single, driving molecular aberration suitable for therapeutic intervention. Conversely, 'crossroads' or 'hub' proteins that oversee multiple, fundamental mechanisms of tumor maintenance may provide broader therapeutic prospects. One such molecule is the molecular chaperone Heat Shock Protein-90 (Hsp90), which is quantitatively and qualitatively exploited in cancer, and controls protein folding quality control in cell proliferation, survival and adaptation. Despite these attractive properties as a cancer drug target, small molecule Hsp90 antagonists have been disappointing in the clinic, with modest or no evidence of patient responses.

Our recent studies may explain this conundrum, and provide a novel molecular foundation for the development of truly effective Hsp90 antagonists. We found that mitochondria of tumor cells, but not most normal tissues, contain an abundant pool of Hsp90 and its related chaperone, TRAP-1. These molecules localize to the mitochondrial matrix, and associate with another mitochondrial chaperone, the immunophilin peptidyl prolyl *cis, trans* isomerase, Cyclophilin D (CypD), which functions as an essential component of the mitochondrial permeability transition pore. The binding of mitochondrial Hsp90 chaperones to CypD antagonizes its pore-forming functions, prevents mitochondrial permeability transition and opposes the initiation of mitochondrial cell death, thus maintaining tumor cell viability.

To target the mitochondrial Hsp90 chaperone network for novel cancer therapeutics, we engineered a novel peptidomimetic antagonist of Hsp90 ATPase function, Shepherdin, to cross the mitochondrial membranes. Shepherdin readily accumulated in mitochondria of tumor cells in vitro and in vivo, and distributed to all submitochondrial compartments, including the intermembrane space, inner membrane, and matrix. Inhibition of mitochondrial Hsp90-directed protein folding by Shepherdin resulted in sudden collapse of mitochondrial integrity, with loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspase-dependent and -independent cell death. Conversely, current Hsp90 inhibitors used in the clinic do not accumulate in mitochondria, and thus do not affect the cytoprotective mitochondrial pool of Hsp90. Consistent with the differential expression of mitochondrial Hsp90 chaperones in tumor, but not most normal tissues, Shepherdin was safe for normal cells and tissues, and did not induce mitochondrial permeability transition or reduce the viability of normal cell types. When tested in various preclinical xenograft models, systemic administration of Shepherdin indistinguishably suppressed the growth of multiple human tumors, without detectable organ or systemic toxicity.

Therefore, selective targeting of the mitochondrial pool of Hsp90 chaperones may provide potent and selective anticancer agents, superior to current, non-subcellularly targeted Hsp90 antagonists.

Keywords: Hsp90, mitochondria, novel anticancer drug

390 Chronic Lymphocytic Leukemia Research Consortium

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The CLL Research Consortium (CRC) is multi-institutional program, investigating the biologic basis of CLL and novel biologic and pharmacologic treatments for this disease. The consortium enables CRC investigators to function beyond the capacities of any single member. It provides for uniform, high-volume sample accrual to a sophisticated national tissue bank, secured on-line information systems, and an infrastructure that facilitates the research on novel therapies and the evaluation of clinical-laboratory relationships that improve clinical staging and/or that assist in the early assessment of response to novel therapies. The CRC has six major projects. **Project 1** (**Carlo Croce, PI**) investigates the genetic basis for CLL and the development of novel probes that can translate genetic discoveries into the clinic. This project has discovered that dysregulation/deletion of microRNAs and ultraconserved non-coding RNAs play a role the pathogenesis of this disease. This is the first human disease for which such regulatory RNAs have been found to play a role. In addition, this project has developed animal models that each recapitulate many facets of this disease. **Project 2** (**John Reed, PI**) investigates the leukemia-cell expression of proteins that regulate susceptibility or resistance to apoptosis and is screening for compounds that can interfere with their biochemical function. This project has identified small molecules that can interfere with the protein machinery that protects leukemia cells from undergoing apoptosis. Some of these agents are undergoing phase I clinical testing in CLL. **Project 3** (**Kipps, PI**) examines for leukemia-associated antigens and novel techniques for active immunotherapy, including gene therapy. This project has identified oncofetal leukemia-associated antigens that are not expressed on normal adult tissues and that appear to play a role in pathogenesis this disease. **Project 4** (**Gribben, PI**) examines the mechanism(s) accounting for ineffective T cell mediated immune response against leukemia-associated antigens and evaluates for specific T cell defects that can interfere with development of effective anti-tumor cellular immune responses. This project has identified mechanisms leading to T-cell anergy and has discovered agents that can reverse the immune-suppressive effect(s) resulting from cognate leukemia-cell:T-cell interaction(s). **Project 5** (**Plunkett, PI**) focuses on promising anticancer agents for phase I/II testing in patients with CLL and examines the activity of these agents either alone or in mechanism-based drug combinations. This project has identified drugs that together have synergistic activity in the treatment of patients with this disease. **Project 6** (**Grever, PI**) is a project that examines pharmacologic inhibitors of key metabolic pathways that are implicated in the pathogenesis of CLL. Furthermore, this project has performed pharmacokinetic/pharmacodynamic studies that have guided development of novel dosing regimens for agents, such as flavopiridol, which have markedly improved the drug's therapeutic activity. The CRC has 4 cores: **Core A** (**Kipps, PI**) is the administrative core that coordinates research investigations, oversees the generation and maintenance of the clinical and laboratory databases and information systems, and facilitates information and data exchange between program participants. **Core B** (**Neuberg, PI**) is the Biostatistics Core that assists investigators in the design and interpretation of basic and clinical research. **Core C** (**Rassenti, PI**) is the Tissue Core that is responsible for tissue banking, sample trafficking, and leukemia-sample laboratory testing and sample-validation. **Core D** (**Wierd, PI**) is the Clinical Trials Core that helps develop, implement, execute, monitor, and analyze CRC clinical trials.

Keywords: chronic lymphocytic leukemia, genetics, immunology, apoptosis, biochemistry, pharmacology

391 Silvestrol: A Novel B-cell Selective Agent for Potential Use in Leukemias and Lymphomas

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Therapeutic options for advanced B-cell malignancies including acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) are limited. Furthermore, available treatments can deplete T lymphocytes, resulting in life-threatening opportunistic infections. Agents with novel mechanisms of action and B-cell selectivity are needed in both diseases. The structurally unique natural product silvestrol was identified through an NCI National Cooperative Drug Discovery Group (Kinghorn, PI). In the NCI 60-cell line screen, silvestrol produces a unique pattern of cytotoxicity suggesting unusual mechanism and efficacy in leukemia. Using primary human leukemia cells, established leukemic cell lines, and animal models, we assessed silvestrol for its potential as a B-cell selective anti-cancer therapy. In CLL patient cells, silvestrol LC_{50} (concentration lethal to 50%) is 6.5 nM at 72 hours. At this concentration, there is no difference in sensitivity of cells from CLL patients with or without the 17p13.1 chromosomal deletion (*p53* site). Both in isolated cells and in whole blood from healthy volunteers and CLL patients, silvestrol is significantly more cytotoxic toward B cells than T cells. Silvestrol causes early reduction in the anti-apoptotic protein Mcl-1 due to translational inhibition with subsequent caspase-independent loss in mitochondrial membrane potential. *In vivo*, silvestrol causes significant B cell reduction relative to T cells in Tcl-1 transgenic mice, and extends survival of 697-SCID mice without discernable toxicity. These data indicate silvestrol has unusual potency and B-cell selectivity both *in vitro* and *in vivo*, and support its development for B-cell malignancies. Due in part to these findings, silvestrol is now undergoing preclinical evaluation by the NCI Developmental Therapeutics Program's Drug Development Group at the Stage IIA level.

Keywords: chronic lymphocytic leukemia, acute lymphoblastic leukemia, natural product

392 Translation of Proteasome Inhibitor-based Combination Regimens Incorporating Agents That Suppress Mitogen-activated Protein Kinase Pathway Signaling Into the Clinic to Enhance Outcomes of Patients With Multiple Myeloma

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The ubiquitin-proteasome pathway is responsible for the vast majority of intracellular proteolysis, and has been validated as a therapeutic target for multiple myeloma through the demonstration of the activity of the proteasome inhibitor bortezomib (VELCADE®) in the up-front and relapsed/refractory settings. Proteasome inhibitors also activate anti-apoptotic mechanisms of chemoresistance, however, thereby limiting their own efficacy, such as through induction of mitogen-activated protein kinase (MAPK) phosphatase (MKP)-1, which suppresses cell death by inactivating c-Jun-N-terminal kinase (JNK). Based on studies showing that anthracyclines specifically repress the MKP-1 promoter, we piloted and validated a regimen combining bortezomib with pegylated liposomal doxorubicin (PLD), which showed enhanced pre-clinical anti-tumor activity. This occurred in association with decreased levels of bortezomib-mediated MKP-1 induction, enhanced levels of activated dually phosphorylated JNK, and increased induction of type I programmed cell death. These findings led to a phase I study of this rationally-designed regimen, which showed that it was both tolerable for patients and effective against their relapsed and/or refractory multiple myeloma. To further test the hypothesis that PLD+bortezomib represented a therapeutic advance, a randomized phase III international study was completed comparing single-agent bortezomib to this two-drug regimen. In this study, PLD+bortezomib induced a superior overall response rate, response quality, time to progression, progression-free survival, response duration, and overall survival compared to bortezomib alone, leading to the approval of this combination by the Food and Drug Administration. Returning to the bench from the bedside, we found that while anthracyclines did indeed suppress MKP-1, this effect was accompanied by greater activity of the p44/42 MAPK pathway, which promotes myeloma cell survival. We therefore considered the possibility that, by addition of an agent that suppresses this pathway, a further increment in anti-tumor activity could be achieved. Since interleukin (IL)-6 is an important cytokine in myeloma that promotes plasma cell growth and survival, and signals in part through p44/42 MAPK, we have more recently been studying the utility of the neutralizing anti-IL-6 monoclonal antibody CNTO 328. By obviating signaling through p44/42, and also through effects on signal transducer and activator of transcription (STAT)-1 and -3, CNTO 328 was indeed found to enhance the activity of single-agent bortezomib. A randomized phase II clinical trial comparing bortezomib to bortezomib with CNTO 328 is currently underway, with the goal of showing that the combination therapy is more active than bortezomib alone. If validated, this would set the stage for evaluation of a regimen containing CNTO 328, PLD, and bortezomib, which would be predicted to be the most active combination from our pre-clinical efforts.

Keywords: proteasome inhibitor, multiple myeloma, doxorubicin

393 Radiolabeled Antibody Therapy for Treatment of Leukemia and Lymphoma

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Division of Clinical Research[1], Fred Hutchinson Cancer Research Center, and the Departments of Medicine[2] and Radiology[3], University of Washington; and Pacific Northwest National Laboratory[4]

The overall goal of this program project grant is to develop effective strategies for treating patients with hematologic malignancies using radiolabeled monoclonal antibodies (Ab) in conjunction with hematopoietic cell transplantation (HCT). Our prior studies have established the feasibility and anti-tumor activity of this approach. We now have refined and extended this approach for both B cell lymphomas and acute leukemias by optizimizing therapy directed at the CD20 and CD45 antigens, respectively. For patients with B cell lymphomas, we describe: 1) a Phase II trial using myeloablative doses of ^{131}I-anti-CD20 Ab with etoposide, cyclophosphamide (CY), and autologous HCT and 2) a more recent study exploring the ability of a synergistic chemotherapeutic agent (Fludarabine; FLU) to enhance the efficacy of ^{131}I-anti-CD20 Ab for older patients. We have also optimized radioimmunotherapy (RIT) for acute leukemia by examining the feasibility, safety and efficacy of administering ^{131}I-anti-CD45 Ab with FLU and total body irradiation (TBI) to patients with advanced AML or MDS undergoing allogeneic HCT, and administering ^{131}I-anti-CD45 Ab with CY, FLU, and TBI to patients with advanced AML or MDS undergoing haploidentical allogeneic HCT. These studies demonstrate that targeted radiotherapy can deliver significantly higher doses of radiation to hematolymphoid organs compared to non-hematolymphoid organs without any significant increase in HCT-related mortality. Finally, we are further refining the delivery of radiation to sites of disease *via* the anti-CD20 and anti-CD45 Abs by development of pretargeted RIT strategies and the use of extracorporeal Ab adsorption therapy to improve the delivery of radiation to target tissues compared to non-target sites. As a first step to translating the pretargeted RIT methodology to human trials, we have performed a series of experiments aimed at identifying the safe, optimal dosing of pretargeted RIT reagents administered to non-human primates. Based on the encouraging results from our pre-clinical studies we are in the process of producing sufficient quantities of pretargeted RIT reagents under good manufacturing practice conditions to pave the way for human clinical trials that we anticipate opening in 2009. We are optimistic that these approaches will improve the cure rates of HCT for patients with lymphoma, acute leukemia and MDS who undergo this procedure each year.

Keywords: radioimmunotherapy, hematologic malignancies, hematopoietic cell transplantation

394 Identification of Inhibitors for MDM2 Ubiquitin Ligase Activity From Natural Products by a Novel High-Throughput Electrochemiluminescent Screen

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High-throughput screening technologies have revolutionized the manner in which potential therapeutics are identified. One casualty of the move to high-throughput screening technologies has been the widespread disappearance of natural product extracts from pharmaceutical screening programs. Though they are the source of lead compounds for ~65% of anticancer and antimicrobial drugs approved by the FDA between 1981-2002, natural products have often been excluded from modern screening programs. This is due, at least in part, to the inherent difficulties in testing complex extract mixtures, which often contain nuisance compounds, in modern bioassay systems. Here we present a novel electrochemiluminescent assay system that is suitable for testing natural product extracts in high-throughput screening systems. To demonstrate its utility, we have developed a screen for inhibition of E3 (ubiquitin ligase) activity utilizing the cancer-relevant molecular target MDM2. Additional screens for inhibition of XIAP, MuRF1 and Nedd4 were also developed to assess specificity. The assays were used to screen over 144,000 natural product extracts. We identified one natural product sempervirine that inhibited MDM2 auto-ubiquitination in vitro and in cells. The compound also inhibited MDM2-mediated p53 degradation, leading to accumulation and activation of p53 in cells. Moreover, sempervirine preferentially induced apoptosis in transformed cells that express wild type p53, suggesting that it could be a potential lead for anti-cancer therapeutics.

Keywords: natural products, high-throughput screening, ubiquitin ligase inhibition

395 Gene Expression Profiling (GEP) Versus Quality of Response Assessment in Myeloma

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Although significant progress has been made in treating multiple myeloma by the introduction of tandem autologous transplants, only 20% of patients enjoy an event-free survival (EFS) in excess of 10 years and have the potential of being cured. Alternatively, 20% of patients on tandem transplant protocols never achieve a complete remission (mainly those who had MGUS or smoldering myeloma prior to their overt myeloma) show no evidence of disease progression at 10 years. Currently available prognostic markers are excellent in identifying patients who are unlikely to have a long survival and are mainly based on genetic features (metaphase cytogenetics and GEP) and extend of disease as assessed by MRI and PET/CT scan. However, it is still impossible to identify patients prior to therapy who have a high likelihood of long EFS. We hypothesize that predicting treatment outcome in myeloma will not only depend on accurate assessment of the remaining tumor load after therapy, but also on genetic characterization of an individual's myeloma cells. Thus, achieving prolonged EFS is dependent on the one hand on adequate tumor cell kill, but also on the genetic make-up of the myeloma cells as it relates to growth and drug resistance. Genetic characteristics related to poor outcome may be present at diagnosis, either in the majority or in a small sub fraction of myeloma, cells or may be acquired during and as a consequence of therapy. To test this hypothesis, we will use samples from two groups of recently diagnosed myeloma patients enrolled on a uniform treatment protocol who represent patients with the worst outcome (those relapsing within two years; estimated population : 25%) and those with a good prognosis (EFS> 4 years; estimated population: 55%). *Specific aim: Identify a GEP associated with high risk of early progression versus a prolonged remission and describe its component genes and molecular pathways; construct validated prediction models that generate both continuous-progression risk scores and low-intermediate-high risk classifications for MM patients.* The underlying hypothesis of this aim is that certain genetic features related to growth kinetics and drug resistance can only be overcome by targeting those genes and pathways associated with poor outcome. GEPs will be performed on CD138-selected plasma cells using the Affimetrix Gene Chip system. RNA amplification will be performed on samples with a small amount of RNA, if at least 1,000 purified plasma cells are available. Data analysis will be based on the Affimetrix 5.1 software. With current treatment approaches we have reached the limits of what can be tolerated. To further improve outcome of myeloma patients, it is critical that we gain a better understanding of the mechanisms of treatment failure and direct therapy specifically towards those genes or gene products characteristic of high-risk myeloma. Our findings should serve as a paradigm for treatment of other hematological malignancies.

Keywords: myeloma, gene expression profiling, treatment

396 Development of HTS Assays for Inhibitors of ERAD and the Ubiquitin Ligase gp78

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There is significant evidence to suggest that part of the efficacy of the proteasome inhibitor, bortezomib, in causing cell death in multiple myeloma derives from preventing myeloma cells from compensating to endoplasmic reticulum (ER) stress with an appropriate unfolded protein response (UPR), resulting in apoptosis. Part of this response includes enhancement of ER-associated degradation (ERAD). Therefore, drugs that selectively block ERAD have the potential to be highly efficacious in multiple myeloma. We have developed a cell-based functional assay that utilizes a chimeric construct of the well-characterized ERAD substrate CD3-delta and yellow fluorescent protein. Inhibition of ERAD will lead to an increase in cellular fluorescence due to failure to degrade this chimeric protein. We have used this assay to screen libraries of pure compounds and of natural product extracts. One potential means of evoking ERAD stress responses would be to inhibit ubiquitin ligases critical to ERAD. One well-characterized ERAD E3 is gp78 (AMFR). Recently we have demonstrated a critical role for the ubiquitin ligase activity of gp78 in sarcoma metastasis by targeting a metastasis suppressor, KAI1, for ubiquitylation and proteasomal degradation. A second, cell-free fluorescence polarization assay is under development to directly evaluate inhibition of the interaction of gp78 with its cognate ubiquitin-conjugating enzyme, Ube2g2. An overview of assay development outcomes and initial screening results will be presented.

Keywords: ubiquitin, myeloma, sarcoma

397 Hsp90 Dependence of Somatically Mutated Epidermal Growth Factor Receptor and ErbB2

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The mature epidermal growth factor receptor (EGFR) neither associates with nor depends on the molecular chaperone Hsp90. Here we show that somatic mutations of EGFR confer Hsp90 dependence. We demonstrate that mutant but not wild-type EGFRs bind to Hsp90 chaperone complexes, and are sensitive to Hsp90 inhibitor-induced degradation. Accordingly, Hsp90 inhibition abrogates downstream signaling from these oncogenic EGFR mutants, suggesting therapeutic promise. This is clinically relevant, as these mutants have been found to play a critical role in non-small cell lung cancer (NSCLC) in that they mediate ultimate resistance to EGFR inhibitors after initial responses. Indeed, the double-point mutant L858R/T790M is resistant to the EGFR inhibitor gefitinib but remains sensitive to Hsp90 inhibition. Furthermore, a subset of EGFR mutants characterized by insertions in exon 20, which as a group confer resistance to EGFR inhibitors, are also sensitive to Hsp90 inhibition. Interestingly, similar mutations in ErbB2, whose wild-type protein naturally depends on Hsp90 function, retain dependence on Hsp90 for stability and downstream signaling capability, and these mutants remain highly sensitive to Hsp90 inhibition. Thus, use of Hsp90 inhibitors should be considered in NSCLC harboring mutated EGFR or ErbB2, especially exon 20 insertion mutants.

Reference: Xu W et al. Brit J Cancer 97:741, 2007.

Keywords: Hsp90 inhibition, epidermal growth factor receptor mutation, ErbB2 mutation, non-small cell lung cancer

398 Molecular and Functional Imaging of Cancer

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The twenty-first century has witnessed an explosion of molecular biology techniques, amazing advances in imaging, and the design of unique imaging probes. Despite the tremendous strides made in these areas of science, the cure for cancer remains beyond our grasp. Cancer is a complex disease and the apparent impenetrability of the disease is largely due to the multiple, often redundant pathways, which appear to evolve through the genetic instability of cancer cells. The ability to identify and image key common pathways specific to cancer cells, and the ability to image the effectiveness and outcome of strategies designed against these targets is critically important in the treatment of this disease. The vision of our JHU ICMIC Program is to combine state-of-the-art imaging capabilities with powerful molecular biology techniques to define strategies with 'intent to cure'. The JHU ICMIC and JHU SAIRP programs have laid a strong foundation for the establishment of a world class *in vivo* cellular molecular imaging program at Johns Hopkins. Our JHU ICMIC structure consists of four interactive and closely related research components focused on hypoxia, HIF-1, and exploiting the hypoxia response element to target cancer cells through choline kinase inhibition. The research components utilize MR, PET and optical imaging technology to understand cancer vascularization, invasion and metastasis, to achieve effective cancer therapy.

The developmental projects are highly relevant to the goals of the ICMIC and interact with the research components. Five resources devoted to administration, molecular biology, imaging, probes, and translational application provide the infrastructure to support the research activities of the ICMIC. A career developmental program is training the future leaders of molecular imaging in cancer. An advisory board consisting of leading scientists at Hopkins, and at several institutions in the US and abroad, provide critical evaluation of the progress made. Strong institutional support and advocacy ensure the fulfillment of its vision.

Keywords: MR imaging, optical imaging, cancer

399 Translational Cancer Prevention Studies: Testing Novel Agents for the Prevention of Estrogen Receptor-negative Breast Cancer

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The successful demonstration that the selective estrogen receptor (ER) modulators (SERMs) tamoxifen and raloxifene reduce the risk of breast cancer has stimulated great interest in using drugs to prevent breast cancer in high-risk women. Results from breast cancer treatment trials also suggest that aromatase inhibitors may be even more effective at preventing breast cancer than are SERMs. However, while SERMs and aromatase inhibitors do prevent the development of many ER-positive breast cancers, these drugs do not prevent ER-negative breast cancer. Thus, there is an urgent need to identify agents which can prevent ER-negative breast cancer. We have studied the cancer preventive activity of several classes of drugs for their ability to prevent ER-negative breast cancer in pre-clinical models and have translated these results and have conducted early phase cancer prevention trials. Results from our preclinical studies demonstrate that rexinoids (analogs of retinoids that bind and activate RXR receptors), tyrosine kinase inhibitors (such as EGFR inhibitors and dual kinase inhibitors that block EGFR and HER2/neu signaling), and cyclo-oxygenase 2 (COX-2) inhibitors all prevent ER-negative breast cancer in transgenic mice. We have tested these agents in early phase cancer prevention clinical trials to determine whether they will show activity in breast tissue and whether they are safe for use in high risk women without breast cancer. We have completed a Phase II cancer prevention trial using the rexinoid bexarotene. In this Phase II double-blind randomized clinical trial, women at high risk of breast cancer (defined as >10% chance of carrying a BRCA1 or 2 mutation) received 200 mg/m^2 bexarotene or placebo for 28 days. Breast core needle biopsies were taken on Days 1 and 29. Pre- and post-treatment samples were assessed for Ki67 expression by immunohistochemistry (primary endpoint) and cyclin D1 expression by qRT-PCR (secondary endpoint). The study was powered to detect a 52% decrease in Ki67 with a planned enrollment of 100. Other secondary biomarkers are still being assessed. Overall, for the primary endpoint, there was no significant change in pre- versus post-treatment Ki67 levels in either arm, and no difference in the change in Ki67 between the two arms (p=0.88). However, there was a significant decrease in post-treatment cyclin D1 RNA levels compared to baseline in both arms (bexarotene arm -46%, p=0.0003; placebo arm -27%, p=0.02). In a subgroup analysis of postmenopausal women, the decrease in cyclin D1 expression was significantly greater in women treated with bexarotene (-65%) than with placebo (-24%)(p=0.03). Thus, while there was no significant reduction in Ki67 staining in the women taking bexarotene, there was a reduction in cyclin D1 expression, particularly in postmenopausal women taking bexarotene, suggesting a biological effect of bexarotene in women at high risk of breast cancer. We are now testing in preclinical studies the cancer preventive activity of drugs that prevent ER-negative breast cancer in combination with hormonal agents such as SERMs. Effective combinations will be translated to early stage cancer prevention clinical trials to test combination preventive therapy in humans. This work was supported by NCI/NIH grants: U19 grant (CA08609)(bexarotene trial), and RO1s (CA10121, CA78480)(preclinical studies).

Keywords: cancer prevention, breast cancer, rexinoid

400 Molecular Targeting of HER2 for Diagnosis and Therapy of Breast Cancer

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Expression of HER2 receptors in breast cancer is correlated with poor prognosis and may be different in distant metastases as compared to the primary tumor. We are developing methods to assess global expression of HER2 *in vivo* and to target HER2 for delivery of therapeutic agents. As the targeting agent we use an Affibody molecule (http://www.affibody.com). These very stable and highly soluble proteins are relatively small (8.3 kDa) and bind to HER2 receptors with high affinity (22 pM). For imaging with PET, SPECT, or optical methods, an appropriate imaging beacon can be attached to a unique C-terminal cysteine residue of Affibody. We used PET imaging with ^{18}F-Z_{HER2}-Affibody to monitor the down-regulation of HER2 following four doses (50 mg/kg) of 17-dimethylaminoethylamino-17-demethoxy-geldanamycin, 17-DMAG, an inhibitor of Hsp90 known to decrease HER2 expression. Animals were scanned before and after treatment. The results were compared with *ex-vivo* analysis of receptor expression. For optical imaging, we used AlexaFluor dyes conjugated with affibody molecules containing an albumin binding domain that extended their circulation time. For therapy, Affibody molecules are conjugated with thermo-sensitive liposomes that can be labeled with beacons for *in vivo* imaging and loaded with therapeutic agents. Alternatively, Affibody molecules are conjugated with gold nanoparticles that could carry relative large amounts of therapeutic agents and, activated with neutrons, would emit gamma radiation allowing *in vivo* monitoring of their distribution by SPECT. In addition, we have recently developed a recombinant DNA construct combining HER2-specific Affibody molecules with Pseudomonas Endotoxin (PE).

Our results showed that Affibody molecules do not affect the targeted cells and that their binding does not interfere with either the binding or the effectiveness of trastuzumab. ^{18}F-Z_{HER2}-Affibody was eliminated quickly from blood and normal tissues, providing high tumor/blood and tumor/muscle ratios by 1h post injection. The signal obtained from PET and optical imaging correlated well with the number of receptors expressed in the studied tumors as assessed by western blot, ELISA, and IHC. Following 17-DMAG treatment, the level of HER2 expression, estimated by PET imaging, in BT474 and Mcf-7/clone18 tumors decreased 70% and 30%. This change was confirmed by the biodistribution studies, ELISA and western blot. The "Affitoxin" molecule (Affibody-PE hybrid) showed dose-dependent HER2-specific toxicity *in vitro*. Preliminary biodistribution studies using AlexaFluor-labeled Affitoxin showed preferential accumulation of the conjugate in the tumor and in the kidney. The efficacy and toxicity of Affitoxins are currently studied.

This strategy, involving assessment of target presence and distribution in an individual patient followed by optimized, target-specific drug delivery, may significantly improve efficacy of breast cancer treatment while reducing side effects.

Keywords: HER2, molecular imaging, targeted therapy

401 Translation of Novel Agents in Early Phase Clinical Trials

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Our UO1 grant, NCI CA70095, is set up to evaluate promising new therapies for patients with malignant disease in a clinically efficient, regulatory-compliant, and scientifically rigorous research environment. Phase I clinical studies of new anti-cancer therapies continue to evolve as basic / translational research has broadened the targeted opportunities to treat malignant disease. Building upon a strong foundation in the conduct of phase I studies, we have assembled an interactive research team that uses rationally designed clinical trials that enhances the molecular / pharmacologic assessment of new drug activity. These trials and assessment methods are designed ultimately to impact on the clinical care of patients diagnosed with cancer. The overall objective of these Phase I/II studies is to determine the appropriate dose and schedule of experimental agents for further evaluation of efficacy in solid tumor /hematologic malignancies and to characterize the acute and chronic toxicities of these anti-cancer therapies. Our Specific Aims are 1) To conduct Phase I clinical trials of novel anti-cancer agents (alone or in combination) in a timely manner; 2) To perform detailed pharmacokinetic studies of these new agents and to correlate these observations with relevant clinical/biologically sound endpoints; 3) To implement correlative and pharmacodynamic laboratory evaluations in proof of drug mechanism phase I clinical trials and to explore optimal relationships between parameters of drug exposure and biological effects; and 4) To maintain a clinical trial infrastructure that is current and compliant with regulatory standards that assure quality care to patients enrolled on early phase clinical trials. Given the scientifically rich environment at the Kimmel Cancer Center at Johns Hopkins, including 7 SPORE grants and many disease-oriented programs within our Cancer Center, we have lead the early phase clinical development of combination strategies employing DNA methyltransferase inhibitors and inhibitors of Histone Deacetylase(HDAC) in both leukemia/myelodysplasia as well as solid tumors such as lung, renal, and prostate cancers. Such early phase trials have led to a phase III cooperative group trial in leukemia, and a phase II trial in non-small cell cancer of the lung. Many of these studies have performed preliminary biomarkers studies of gene expression from patient derived cellular material. Direct from SPORE sponsored research, we have conducted novel combinations studies of inhibitors of histone deacetylase in combination with estrogen receptor targeted therapies, and combination studies of various antiangiogenic and immunotherapeutic agents in combination with HDAC inhibitors. Another area of strength is that of signal transduction pathways. In translating concepts from our Head and Neck Spore, we have received approval to conduct a phase I study of a novel MEK inhibitor in combination with an insulin-growth factor receptor monoclonal antibody. Our studies, led by Judy Karp, in conjunction with investigators at the Mayo Clinic, have advanced our understanding of the farnesyltransferase inhibitor tipifarnib in adult leukemias. We are also conducting studies of the PARP inhibitor ABT888 in combination with topotecan, incorporating analytical studies at our site with pharmacodynamic studies conducted at the NCI. Our research group remains flexible to expansion and to explore other targets of interest / trial designs as well as well-positioned to further incorporate novel imaging into our early phase clinical trials. For the last four years, we have averaged 140 new patients participating in our NCI phase I grant. Scientifically, members of our program actively participate in the Investigational Drug Steering Committee (IDSC).

Keywords: early phase clinical trials, epigenetics, signal transduction

402 Correlative Molecular Endpoints From RTOG 0211: Phase I/II Study of Gefitinib + Radiation for Newly Diagnosed Glioblastoma(GBM)

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Background: RTOG 0211 revealed that the addition of Gefitinib to radiation was well-tolerated , but survival was not significantly improved compared to historical controls (ASTRO 2006). The present report is the first to evaluate molecular correlates of clinical outcome in newly diagnosed GBM treated by anti-EGFR therapies and represents the most comprehensive correlative analysis performed on GBM patients treated by anti-EGFR therapies.

Methods: Tissue blocks were prospectively collected on 74 out of the 148 RTOG 0211 cases, which were used to generate tissue microarrays (TMAs). The predictive values of 12 molecules integral to EGFR signaling either have been examined or will be examined by the time of the meeting (EGFR, pEGFR, EGFRvIII, PTEN, pAKT, pMAPK, pmTor, IGFR1, NFKB, Survivin, MGMT, and pSrc). The molecules that have been examined to-date include EGFR, EGFRvIII, PTEN, and pAKT. The analysis was performed using the Histo-Rx AQUA platform. In addition, EGFRvIII and PTEN were also analyzed using traditional immunohistochemical staining and manual scoring to reproduce the technique that was used in the Mellinghoff NEJM report.

Results: Neither total EGFR, EGFRvIII, nor PTEN expression as single markers were significantly associated with either overall (OS) or progression-free survival (PFS) in GBM patients treated on RTOG 0211. Patients with co-expression of EGFRvIII and intact PTEN as determined by manual scoring had 12-month OS and 5.4-month PFS, compared to 9.5 months OS and 4.7 months PFS in non-co-expressing patients [OS: HR=1.462 (95% CI: 0.689-3.10); PFS: HR=1.836 (95%CI: 0.797-4.23), p=NS (study underpowered to detect significant difference)]. Likewise, AQUA scoring failed to find any significant correlation with outcome in co-expressors of EGFRvIII and PTEN. Patients expressing high versus low levels of pAKT had significantly shorter survival times (p=0.047).

Conclusions: In the upfront setting, activation of AKT signaling appears to be associated with adverse outcome in Gefitinib-treated GBM patients. The complete RTOG 0211 correlative data based on the 12-marker panel will be presented at the time of the meeting.

Keywords: glioblastoma, radiation, EGFR

403 Imaging Cancer Treatment Response: PET Translational Research at the University of Washington

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Imaging scientists and cancer doctors at UW have been developing prognosis and prediction methods by characterizing the biochemistry of cancer. Our methods use Positron Emission Tomography (PET) imaging in a multi-disciplinary UW/FHCRC cancer program that has led the way in understanding how to use this powerful molecular imaging modality in applications to cancer. In addition to 3D imaging capabilities to assess heterogeneity, PET provides noninvasive quantitative measurement of tissue concentrations of biologically relevant radiotracers in any body region. Our focus is on general characteristics of the tumor phenotype and traits that might lead to resistance to treatment interventions. Our approach uses knowledge of cancer biology to target pathways that have broad scientific validity, address important clinical needs such as choosing treatment options or assessing treatment response, and are feasible from an imaging perspective. These capabilities have led to development of imaging agents more specific to cancer biology than glycolysis measured by FDG. We image cellular proliferation with C-11 thymidine and F-18 fluorothymidine and cell membrane synthesis using C-11 acetate and are developing F-18 fluoroannexin V for measuring cellular death. Imaging can also be used to assess the spatial heterogeneity and/or time course of resistance factors such as hypoxia (F-18 misonidazole), multiple drug resistance via P-glycoprotein (C-11 verapamil) or estrogen receptor status (F-18 16-α-fluoroestradiol). Other new imaging agents include labeled chemotherapeutics. The use of PET imaging requires development of analysis algorithms for the time course of uptake of each tracer so that delivery can be distinguished from local utilization. For example uptake of proliferation tracers is often dominated by blood flow and a calculated image is needed to measure binding in the salvage pathway versus non-incorporated tracer.

Currently, the group uses combinations of PET agents to probe cancer biology in different histologic types. Imaging for planning treatment for the individual patient or as a pharmacodynamic endpoint are two ways experimental imaging procedures are being used to optimize experimental treatments. In this setting, imaging procedures are validated as reflecting biochemical processes rather than as predictors of clinical outcome. Much of our research is focused on imaging for identifying and understanding cancer treatment responses. Highlights from our recent studies include:

- New parametric models for imaging tissue proliferation can distinguish proliferation from transport and can be used to distinguish radionecrosis from recurrence of brain tumors.
- Hypoxic tumor volume in glioblastoma and HNSCC correlate with survival. Hypoxia images can be used to define a biologic target volume for an image-guided radiotherapy boost.
- Response to anti-vascular therapy can be observed as a change in hypoxia images.
- Estrogen receptor expression can be heterogeneous within an individual and the dose required to saturate ER in tumors can be higher than in normal uterus.
- Multi-tracer studies are feasible to quantify and distinguish resistance factors in patients with sarcomas.

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Keywords: PET imaging, pharmacodynamic endpoints, treatment resistance

404 Normalization of the Tumor Microenvironment for Therapy: Bench to Bedside and Back

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In the past 8 years, our Program Project team has used a systems approach to overcome the vascular and interstitial barriers impeding delivery and efficacy of molecular medicine. Our most exciting and clinically relevant finding is that when the balance of pro- and anti-angiogenic molecules is partially restored in tumors by VEGF blockade, the aberrant structure and function of the vasculature and interstitial matrix transiently become closer to that of normal tissue. Referred to as vascular normalization, this concept has generated a new paradigm in the field of anti-angiogenic therapy and provided an explanation of how antiangiogenic therapy enhances chemo- and radiation therapy. We have tested this concept in rectal carcinoma patients receiving the anti-VEGF antibody bevacizumab (Avastin) with chemo- and radiation therapies, and our clinical findings mirrored those made in our transplanted tumor models in Project 1 (Willett et al., *Nat Med* 2004). Several independent laboratories have published data in support of our findings on vascular normalization. Most importantly, our work has spawned multidisciplinary clinical studies of antibodies or tyrosine kinase inhibitors that target VEGF and/or PDGF pathways for glioblastoma, sarcomas, breast, liver, ovarian, head and neck patients at the MGH.

In our Program Project, we address critical issues in the clinical translation of anti-angiogenic therapy of tumors. The overall theme is to characterize tumor response to antiangiogenic agents—currently in clinical testing—that differentially target the VEGF and PDGF signaling pathways. Understanding the effect of each agent on vascular normalization will allow the design of specific regimens for the combination of cytotoxic therapies with each of these antiangiogenic agents. In Project 1, the goal is to improve these treatments by manipulating perivascular cell recruitment. We found that normalization of tumor vasculature by improved perivascular cell support makes it more efficient for oxygen delivery and thus, enhances tumor response to radiotherapy (Kashiwagi et al., *Nat Med* 2008). In addition, we aim to establish a "normalization index" for each antiangiogenic agent, determine drug delivery and tumor response and complement studies in Project 1 by evaluating surrogate markers of biological response (circulating proteins and progenitor cells) in Project 2. We established that normalization of vascular function can decrease edema in brain tumor models, prolonging survival (Kamoun et al., submitted). Finally, we are studying the role of cytokines and proteases in the permeabilization of the collagen matrix in tumors, with the aim of using agents that modify extracellular matrix to improve gene therapy in Project 3. We discovered that degradation of fibrillar collagen increases the distribution and efficacy of oncolytic viruses (McKee et al., *Cancer Res* 2006 and Mok et al., *Cancer Res* 2007). Each Project relies upon unique *in vitro* and *in vivo* models, powerful intravital techniques, innovative imaging technologies, mathematical modeling and statistical support (Core A); cutting-edge molecular, cellular, and histological expertise (Core B); superb surgical and animal support (Core C); and administrative support (Core D). We are testing the preclinical studies in blood and tissue obtained from clinical trials. With this infrastructure, and the help of our clinical collaborators, we intend to develop optimal strategies for cancer therapy and translate these scientific discoveries in clinical studies.

Keywords: normalization, tumor microenvironment, drug distribution

405 Validation of Genomic Targets in Melanoma

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Recent cDNA microarray analyses of nevi, primary and metastatic melanomas performed in our laboratory have identified a large number of genes whose level of expression can be used to distinguish between known stages in the tumor progression cascade of melanoma. To date, we have confirmed the utility of several of the genes suggested by that analysis as novel biomarkers for melanoma. To accomplish this, we created a tissue microarray (TMA) of over 350 primary melanoma specimens with either two years of follow up, first relapse, or undergoing sentinel lymph node (SLN) biopsy. Among the genes overexpressed in our analyses were NCOA3 (nuclear receptor coactivator 3), SPP1 (osteopontin), and RGS1 (regulator of G signaling protein 1). We assessed the prognostic significance of NCOA3, SPP1, and RGS1 expression using immunohistochemical analysis of the TMA. For each of the markers, marker overexpression was significantly predictive of SLN metastasis. Kaplan-Meier analysis demonstrated a significant association between marker overexpression and reduced relapse-free (RFS) and disease-specific (DSS) survival. Logistic regression analysis revealed marker expression to be an independent predictor of SLN status. Multivariate Cox regression analysis showed that marker expression independently predictive of DSS. A multi-marker index combining the expression level of the three markers was developed for its ability to predict various outcomes associated with melanoma. Increasing multi-marker index scores were significantly predictive of SLN metastasis and reduced RFS, DMFS (distant metastasis-free survival), and DSS. Multivariate regression analyses revealed multi-marker expression scores to be an independent predictor of SLN status, RFS, DMFS, and DSS. The multi-marker index was the most powerful factor predicting DSS, and remained so even after including SLN status in the multivariate model. The multi-marker index was independent of AJCC stage in predicting DSS. Systemic ribozyme and siRNA-based targeting of several of the markers identified by the gene expression profiling analyses suppressed the metastatic progression of melanoma in murine models. These studies have identified novel biomarkers for melanoma and novel targets for therapy of melanoma metastasis.

Keywords: melanoma, biomarker, tissue microarray

406 A Comparative Approach to Cancer Biology and Therapy

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Opportunity: There is a unique opportunity to include naturally occurring cancers that develop in pet dogs, as translational models, in the development path of new human cancer drugs. Naturally occurring cancers in pet dogs and humans share many features, including histological appearance, tumor genetics, molecular targets, biological behavior and response to both conventional and novel targeted cancer therapies. Indeed, the formal integration of studies that include pet dogs with cancer has now begun and is becoming a more common part of an innovative cancer drug development process.

Background: The long history of dogs in biomedical research, their strong anatomic and physiologic similarities to humans, and the number of pet dogs that are diagnosed and managed with cancer each year (United States est. 1 million per year) supports the potential translational value of new cancer evaluation in large and outbred animals. Cancers developing in these animals are naturally occurring, with the tumor, the host and the tumor microenvironment all being syngeneic. Tumor initiation and progression are influenced by similar factors in both human and canine cancers, including age, nutrition, sex, reproductive status, and environmental exposures. The spectrum of cancers seen in pet dogs is as diverse as the cancers seen in human patients. Not surprisingly the genetic events that are understood to be associated with cancer development and progression in humans are the same as those that occur in canine cancers. The biological complexity of cancers in pet animals is high and emerges from a similar intra-tumoral (cell-to-cell) heterogeneity seen cancer in human cancer patients. A natural consequence of this heterogeneity is the acquisition of resistance to therapy, recurrence of disease, and metastasis to distant sites.

Since there are no gold standard treatments for pet animals with cancer, new cancer treatments can be provided to pet dogs with cancer at earlier stages of progression than human trials. Flexibility in the conduct and design of trials that include dogs with cancer permits serial biopsy of tumor and collection of biological fluids, and imaging endpoints during exposure to novel cancer agents. Lastly, the rates of cancer progression are notably faster in pet dogs than humans, accordingly these studies can be completed without interruption to the existing development path.

Implementation: In an effort to develop this novel cancer drug development opportunity, address potential risks with this approach and to establish the organizational infrastructure to undertake translational clinical trials in pet dogs, the National Cancer Institute's Center for Cancer Research has recently launched the Comparative Oncology Program. Through the NCI Comparative Oncology Program a multi-center consortium of veterinary colleges (COTC: Comparative Oncology Trials Consortium) have begun preclinical studies including pet dogs with cancer. These studies have been initiated and integrated within the preclinical and clinical development path for new cancer drugs. The COTC trials are based on collaboration and partnerships between academic institutions, the NCI and the pharmaceutical industry. We expect that studies that include pet dogs with cancer will inform and improve the development of new cancer drugs through answers to many questions not currently answered by conventional preclinical and early human clinical trials.

Keywords: preclinical studies, drug development, translational model

407 Combinatorial Therapy With Signal Inhibitors in Series: Clinical Trial and Proof of Concept Translational Studies

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We have focused on the concept that combinatorial therapy using molecular modifiers in series or in parallel will result in optimal reduction in key molecular pathway function. We began by investigating a multi-active and a focused activity tyrosine kinase inhibitor, imatinib and gefitinib, respectively, in ovarian cancer. Ovarian cancer is known to have expression of the targets and to have had these targets linked to outcome. Serial biopsies were taken to allow measurement of changes in target proteins, either direct targets, such as abl, kit, and EGFR, or downstream effector proteins, such as AKT and MEK/ERK. Reverse phase protein arrays were used to measure the total and activated (phosphoryated) protein. Lack of clinical activity precluded linking target modulation with outcome, however, both studies included prospective objectives for analysis of target modulation and toxicity. Such links were shown, demonstrating target modulation correlation with gastrointestinal and skin toxicity. Those results led to three possible explanations: 1) the target is not important in the tumor biology; 2) the target is important but its modulation was not sufficient; 3) the target is important and sufficiently inhibited, but this effect can be overcome by paracrine or parallel signaling to the same downstream targets. This led to our next hypothesis, that rational combination of targets can overcome these potential obstacles. The combination of sorafenib, a promiscuous raf-1/VEGFR2 kinase inhibitor, with bevacizumab was selected to block VEGF, as it is a potent pro-angiogenic cytokine and is known to be upregulated by TKIs including sorafenib, to block the VEGF receptor, and to block a central downstream effector, Raf. This was further designed to alter signaling in the tumor microenvironment, targeting activated vasculature, activated stromal components, as well as the tumor. It was hypothesized that this would modify both the survival and invasion behaviors of the cells. Our final recommended dosing after different test schedules and doses is sorafenib 200mg/d (may be after a 2 mo lead in of 200mg bid) with 5mg/kg bevacizumab every other week. A surprising clinical activity was observed in epithelial ovarian cancer patients stimulating the phase II trial that is currently ongoing. Translational endpoints were applied to the phase I study in a unique design. Patients were randomized between the different agents as monotherapy for the first month to allow translational endpoints to be done under single agent v. combination exposure. The endpoints include: dynamic imaging using FDG-PET and DCE-MRI, and biopsies to measure endpoints in tumor tissue by reverse phase protein array, all done pre therapy, 2 weeks into monotherapy and 2 weeks into combination therapy (6 weeks of total therapy), monthly blood collections for measure of pro-angiogenic cytokines, collections for pharmacokinetics, pharmacogenomics, and mutational analysis of Raf-1 and H-Ras. These were designed to address proof of target modulation and to test proof of the concept of combinatorial therapy to link to clinical outcomes of tumor effect and toxicity. We believe that this is an example of rational design for hypothesis-driven drug combination, modification of clinical trial design within the phase I context to allow broader hypothesis-testing, application of an array of translational endpoints incorporating different modalities to potentiate the opportunity for interpretable results, and to optimize identification of predictive, prognostic, or selective biomarkers to be taken into phase II study.

Keywords: ovarian cancer, anti-angiogenesis, biochemical combination therapy

408 Strategies for Phase I Development of Targeted Anti-Cancer Agents

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The first step in clinical drug development is the phase I trial. Information garnered from these trials impacts all further development of the drug. Some basic premises that can be recognized in phase I trials include:

- The optimal dose/schedule for the drug needs to be determined. Toxicity, response, and biologic activity are often not predicted from preclinical studies.
- Pharmacokinetics/pharmacodynamics are crucial for optimizing dosing.
- Regimens should be tailored to individual tumor types. Strategies successful for hematological malignancies may not be successful for solid tumors. For instance, the MTD in hematologic malignancies is often considerably higher than that in solid tumors because, at least with cytotoxic agents, significant myelosuppression may be part and parcel of the response.
- The optimal biologic dose (OBD) for moving from phase I to phase II studies with targeted agents may be the dose at which maximum interaction between the agent and its target occurs. This OBD may or may not be the same as the maximum tolerated dose (MTD).
- Determination of optimal interaction between the drug and target requires correlative studies: molecular/cellular endpoints and/or functional imaging.
- The activity and toxicity of an individual agent may differ from that when a $\geq$2 agent are combined. Combinations of agents may be superior to individual agents as their interaction may make tumor eradication more likely.

Over the last five years, a total of over 25 active phase I studies of targeted anti-cancer agents have been performed under the auspices of our UO1. Targets of these agents include but are not limited to histone deacetylase, farnesytransferase, angiogenesis, mtor, and raf kinase, and insulin growth factor receptor. Over 350 patients have been entered onto these trials. Our experience indicates that, in addition to the above, identifying response signals in Phase I studies should be an important objective for these studies, since such responses are excellent predictors of response in follow up Phase II efficacy studies.

Keywords: phase I cancer drug development, targeted therapy, anti-cancer agents

409 Discerning Mechanisms of Action and Biomarkers for Dasatinib Through Combined Chemical and Shotgun Phosphoproteomics

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Discerning mechanisms of multi-targeted tyrosine kinase inhibitors such as dasatinib can be complex given the complexity of SRC family kinase signal transduction, cell context dependent expression patterns of kinases and their substrate proteins, and the numerous tyrosine and non-tyrosine kinases potentially interacting with dasatinib. Using lung cancer cell lines we identified the signaling pathways downstream of the SRC inhibitor dasatinib in lung cancer cells. We describe a strategy to comprehend signaling pathways active in lung cancer cells targeted by dasatinib employing drug-affinity matrixes to identify direct interacting kinases combined with immunoaffinity purification of tyrosine phosphorylated peptides corresponding to activated tyrosine kinases. Dasatinib could inhibit cell proliferation and invasion in lung cancer cell lines which was associated with changes in downstream ERK, Akt, Stat3, and FAK signaling. Using dasatinib as bait for drug-affinity chromatography, we reproducibly identified more than 40 different kinases expressed in lung cancer cell extracts. Approximately half of these targets are tyrosine kinase including Src family members (LYN, SRC, LCK, YES), non-receptor tyrosine kinases (FRK, BRK, and ACK), and receptor tyrosine kinases (Ephrin receptors, DDR1, and EGFR). Using immunoaffinity purification of tyrosine phosphorylated tryptic peptides we identified peptides in lung cancer cells corresponding to the autophosphorylation site of most of these direct tyrosine kinase targets. We found that dasatinib decreased autophosphorylation tyrosine sites of these targets in a concentration-dependent manner and could inhibit tyrosine phosphorylated peptides corresponding to known c-SRC substrates. Examination of a phosphopeptide database characterizing tyrosine phosphoryalted peptides in human lung cancer specimens reveal a large number of these targets are expressed and thus relevant targets for dasatinib in lung cancer. The overall approach described here can help understand mechanisms of multi-targeted tyrosine kinase inhibitors by using MS-based approaches to determine direct kinase targets, expression in tumor cells, and modulation by drug. This knowledge can suggest rationale combination strategies and potential biomarkers to select patients likely to benefit from dasatinib therapy.

Keywords: Dasatinib, lung cancer, tyrosine kinase inhibitor

410 Understanding the Development of Human Bladder Cancer by Using a Whole-Organ Genomic Mapping Strategy

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The search for the genomic sequences involved in human cancers can be greatly facilitated by maps of genomic imbalances identifying the involved chromosomal regions, particularly those that participate in the development of occult preneoplastic conditions that progress to clinically aggressive invasive cancer. The integration of such regions with human genome sequence variation may provide valuable clues about their overall structure and gene content. By extension, such knowledge may help us understand the underlying genetic components involved in the initiation and progression of these cancers. We describe the development of a genome-wide map of human bladder cancer that tracks its progression from in situ precursor conditions to invasive disease. Testing for allelic losses using a genome-wide panel of 787 microsatellite markers was performed on multiple DNA samples, extracted from the entire mucosal surface of the bladder and corresponding to normal urothelium, in situ preneoplastic lesions, and invasive carcinoma. Using this approach, we matched the clonal allelic losses in distinct chromosomal regions to specific phases of bladder neoplasia and produced a detailed genetic map of bladder cancer development. These analyses revealed three major waves of genetic changes associated with growth advantages of successive clones and reflecting a stepwise conversion of normal urothelial cells into cancer cells. The genetic changes map to six regions at 3q22–q24, 5q22–q31, 9q21–q22, 10q26, 13q14, and 17p13, which may represent critical hits driving the development of bladder cancer. Finally, we performed high-resolution mapping using single nucleotide polymorphism markers within one region on chromosome 13q14, containing the model tumor suppressor gene RB1, and defined a minimal deleted region associated with clonal expansion of in situ neoplasia. These analyses provided new insights on the involvement of several non-coding sequences mapping to the region and identified novel target genes, termed forerunner (FR) genes, involved in early phases of cancer development.

Keywords: forerunner (FR) genes, whole-organ histologic and genetic mapping, high resolution mapping with SNPs

411 California Cancer Consortium with Pittsburgh and Penn State: NCI-Supported Infrastructure for Molecular and Clinical Pharmacodynamic Phase I and II Trials

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The Phase I Cooperative Agreement (U01 CA062505) of the California Cancer Consortium (*CCC*) continues a longstanding multi-institutional collaboration among 3 NCI-designated Cancer Centers: the City of Hope, the University of Southern California, and the University of California at Davis, with extensive experience in the conduct of U01-sponsored clinical trials. The Phase II Contract (N01 CM62209) is collaboration between *CCC* and a pair of institutions with a separate Phase I Cooperative Agreement (U01 CA099168): the University of Pittsburgh, and Pennsylvania State University. The specific aims for the *CCC* U01 are: (1) to perform Phase I clinical, pharmacokinetic, and pharmacodynamic trials of NCI-sponsored compounds in concert with molecular evaluation and target assessment of these effects in tumor cells and surrogate tissues; (2) to examine in special patient populations the clinical pharmacology of targeted anticancer agents whose therapeutic effects may be altered because of abnormal organ function or because of inherited differences in genes controlling drug disposition and activity; and (3) to identify new and informative laboratory correlates of biologic activity and drug resistance and explore novel functional endpoints of tumor response, progression, and clinical benefit for patients entered on the clinical trials of the CCC. The specific aims for the *CCCP* N01 are: (1) to rapidly initiate and complete laboratory-based early clinical trials evaluating the safety and efficacy of NCI-sponsored agents, alone or in combination, maximizing the patient and scientific resources of an established consortium with demonstrated expertise in clinical drug development; (2) to employ a systematic approach to collection, processing, and storage of blood, normal and tumor tissue, and other relevant biologic samples for molecular and pharmacologic studies; (3) to investigate the pharmacokinetics, pharmacodynamics and pharmacogenomics of NCI-sponsored agents under study, including evaluation in special patient populations, such as those with impaired end-organ function, the elderly, and racial/ethnic groups in whom differences are anticipated; (4) to evaluate the biologic effects of novel therapeutics (including cytostatic agents) on their molecular targets, to incorporate appropriate study designs and statistical methods to assess intermediate endpoints (including functional imaging) and to correlate findings with clinically relevant outcomes; and (5) to utilize scientific expertise in the areas of a) DNA metabolism, b) microtubule function, c) signal transduction and cell cycle regulation, and d) tumor angiogenesis, in order to assess drug-target interactions, and to optimize laboratory and imaging correlative studies.

From a more global perspective, the investigators of the *CCCP* have demonstrated the ability to complete pharmacodynamically-driven Phase I and II trials in a multi-site consortium. In addition to conducting trials of agents under NCI-sponsored INDs, we have conducted trials of two investigator-sponsored IND agents developed under the DTP RAID program, and have the capacity to incorporate informative correlative studies developed by laboratory investigators outside of the *CCCP*. In a recent four-year period, 226 patients were accrued to *CCC* U01-sponsored trials and 764 patients were accrued to *CCCP* N01-sponsored trials. Over 5000 assays were performed on blood, urine, and tissue samples collected by the *CCC* Pharmacology Core Facility. The simultaneous pursuit of both Phase I objectives by *CCC* and II objectives by *CCCP* has facilitated the transfer of pharmacologic expertise among the institutions as well as the translation of novel laboratory observations from the Phase I to the Phase II setting.

Keywords: Phase I, Phase II, pharmacodynamics

412 NSABP Biospecimen Bank

Soonmyung Paik, **Norman Wolmark**

The National Surgical Adjuvant Breast and Bowel Project (NSABP)

The NSABP has randomized more than 100,000 patients diagnosed with breast or colorectal cancer into clinical trials over the past 50 years. Based on incremental benefit achieved through multiple steps of evolution of treatment modalities tested in those trials, significant improvement in patient outcome has been achieved. However this also means that there is a significant over-treatment of patients who did not benefit from the various interventions. Development of reliable prognostic and predictive tests is required to deliver the promise of personalized treatment. Archived tumor tissue blocks from historical trials provide an essential resource to develop such tests since trials cannot be conducted again once efficacy of newer regimens is demonstrated due to ethical reasons.

The NSABP Biospecimen Bank serves as an open resource to the scientific community to provide tissues for development and clinical validation of new prognostic or predictive tests. Tissue specimens are provided to investigators from both academia and the private sector based on scientific merit.

Studies for prognostic or predictive tests are conducted with statistical rigor which requires the validation of the candidate algorithm in a completely independent clinical cohort that was not used for the development of the algorithm.

Gene expression profiling using QRT-PCR in collaboration with Genomic Health, Inc., has led to the development of OncotypeDx assay based on expression levels of 21 genes. This assay provides continuous estimates of risk of distant failure for patients diagnosed with estrogen receptor positive node negative breast cancer who are treated with tamoxifen and provides guidance as to the need for chemotherapy in addition to tamoxifen. A similar assay for colon cancer is undergoing validation.

In-house research efforts have resulted in development of methods for microarray gene expression profiling of formalin fixed paraffin embedded tumor tissue. The method is currently being utilized to discover predictors of the degree of benefit from trastuzumab added to adjuvant chemotherapy.

Keywords: tissue bank, gene expression, correlative science

413 Early Clinical Trials Focused on Biomarkers

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Early clinical trials generally focus on drug development, using the classical paradigm of phase I testing to define the maximally tolerated dose, followed by phase II testing to measure the response rate at that dose. Although our phase I portfolio includes a number of classical phase I trials, we have conducted a number of studies that have focused on the development of biomarkers to predict toxicity and/or guide dosing of a particular agent. Two such studies have been completed and published, relating to irinotecan and erlotinib, respectively. A third study of sorafenib is ongoing.

The two published studies were prospectively designed to test the relationship between a candidate polymorphism and toxicity. The first study (Innocenti, J Clin Oncol, 2004), of the relationship of the *UGT1A1*28* promoter indel polymorphism to irinotecan-induced neutropenia, was the primary basis of a change to the Pfizer label for irinotecan (brand name, Camptosar), approved by the FDA in 2005. This label change included a warning regarding the increased risk of neutropenia in patients homozygous for this polymorphism, when the standard dose (350 mg/m^2) of irinotecan is administered.

The second study (Rudin, J Clin Oncol, 2008) was designed to test the relationship of polymorphisms in the *EGFR* promoter (Liu, Cancer Res, 2005) and intron 1 (Liu, Clin Cancer Res, 2003) to the skin and gastrointestinal toxicity of erlotinib. Variability in skin rash was best explained by a multivariate logistic regression model incorporating the trough erlotinib plasma concentration and the *EGFR* intron 1 polymorphism. Variability in diarrhea was associated with the two linked polymorphisms in the *EGFR* promoter, but not with erlotinib concentration. In addition, there appeared to be an association between erlotinib exposure and two new variants in the *ABCG2* promoter, suggesting that variability in expression of the ABCG2 transporter may have a major impact on the pharmacokinetics of this agent.

The third study of sorafenib is ongoing, and is designed to test the hypothesis that higher doses of sorafenib will lead to a greater effect on a potential biomarker for VEGF inhibition, ambulatory blood pressure. Previous studies from us and others have demonstrated that an increase in blood pressure is a mechanism-related toxicity of VEGF inhibitors. Thus, we have suggested that ambulatory blood pressure could be monitored to optimize dose and/or schedule of these agents. Our ongoing trial evaluates the impact of higher daily doses of sorafenib (400 mg bid vs. 400 mg tid vs. 600 mg bid) on the pharmacokinetics and pharmacodynamics of this agent. If a greater increase in blood pressure can be obtained with a higher dose, then this would support further randomized trials of higher doses vs. the standard 400 mg bid dose.

Keywords: pharmacogenetics, biomarker, blood pressure

414 Identification of Predictors of Response in a Phase II Trial of Preoperative Cis-platinum in Early Stage Triple-Negative Breast Cancer

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Laboratory data suggested that tumors deficient in BRCA1 may be more susceptible to the DNA damaging agent cis-platinum. Genomic characterization demonstrated marked similarities between BRCA1-associated and sporadic triple-negative breast tumors (negative for estrogen receptor (ER), progesterone receptor (PR) and HER2). In a related study, ΔNp63 was found to control a pathway for TAp73-dependent cisplatin sensitivity that was specific to triple-negative breast tumors. We conducted a phase II trial of preoperative cis-platinum in 28 patients with early stage triple-negative breast cancer. Pathologic response was measured using the Miller-Payne (M-P) system based on radiologic-pathologic assessment of tumor reduction after chemotherapy. Six of 28 (22%) achieved pathologic complete response (M-P score 5), including both carriers of germline BRCA1 mutations. Overall, 14 (50%) were sensitive (M-P scores 3,4,5), and 14 (50%) were resistant (M-P 0,1,2) to cisplatin. Frozen tumor tissue obtained prior to initiation of chemotherapy was available for 24 of the 28 patients. Assays were performed for germline BRCA1 mutation, tumor BRCA1 expression and promoter methylation, p53 mutation, and ΔNp63/TAp73 expression ratio. Pretreatment tumor samples were also analyzed by gene expression array profiling and MIP array DNA profiling.

Hierarchical clustering of gene expression profiles show that 23 of 24 tumors cluster with other basal-like tumors in a reference cohort. One case clustered with HER2 positive tumors and was resistant to cisplatin; this case was HER2 2+ by IHC and FISH not amplified. Supervised learning identified no single gene with significant association with cisplatin response after Bonferroni correction for multiple comparisons. Published gene signatures for E2F3 overexpression, higher chromosomal instability, and proliferation were weakly associated with cisplatin sensitivity.

Sequencing confirmed known germline BRCA1 mutations,in two patients, and no additional germline mutations were identified. RT-PCR measurement showed a trend for lower BRCA1 expression levels in sensitive tumors and combination of low BRCA1 and high Ki67 expression identifies 7/11 sensitive and 1/10 resistant tumors (chisq 6.39, $p = 0.012$). BRCA1 expression is correlated with expression of neighbor of BRCA1 gene 2 (NBR2) which shares the same promoter region, suggesting alteration of the promoter may underly the reduced expression levels.

P53 sequence demonstrated 6 wild type, 11 substitution mutations, and 5 truncating mutations. Truncating p53 mutations were significantly associated with sensitivity to cisplatin. A ratio of ΔNp63/ TAp73 > 2 (biomarker positive) was found in 6/11 sensitive and 2/10 resistant tumors (chisq 2.65, $p=0.1$) and the p63/p73 biomarker was positive in 3/4 tumors with pathologic complete response, supporting a possible trend for association with cisplatin sensitivity.

In summary, we have clinical evidence to suggest low BRCA1/high Ki67, ΔNp63/ TAp73 > 2, and p53 truncating mutations are biomarkers associated with cisplatin sensitivity. These will be tested in a second cisplatin-based preoperative treatment trial that is currently underway.

Keywords: triple-negative, cis-platinum, response biomarkers

415 Creating an Academic Drug Discovery Environment

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Drug discovery in academia has traditionally been limited to the identification of potential therapeutic targets for a number of reasons. In addition to the high cost, developing new therapeutic approaches to treating human disease cuts across traditional scientific disciplines, involves the integration of both biological and chemical research and needs to be informed by the knowledge of physicians of what is possible and practical for manipulating human physiology. The traditional ways of apportioning credit for academic discoveries, the demands to publish rapidly and often, and difficulty of communicating across disciplines all work against the integration required for drug discovery efforts.

UT Southwestern has an active research program in therapeutic drug discovery that includes synthetic organic chemistry, natural products chemistry, functional genomic studies probing for novel therapeutic targets and high-throughput screening of small molecule libraries. Key to the success of our drug discovery efforts is the creation of core laboratories staffed by expert, non-tenure track researchers who support the efforts of our academic scientists and a bimonthly Cancer and Chemistry meeting that brings together basic scientists and clinicians to discuss current drug discovery projects. We have established a high-throughput screening (HTS) laboratory equipped to screen genomic siRNA libraries or a library of 200,000 synthetic organic compounds. Screening efforts are supported by a medicinal chemistry laboratory that conducts studies of structure-activity relationships required to improve the properties of compounds of interest. A preclinical pharmacology laboratory supplies the pharmacokinetic and pharmacodynamic studies required to develop compounds with properties suitable for use in animals and tests those compounds for toxicity and efficacy using mouse xenograft models. Successful HTS projects have had two pathways towards clinical trials. Investigators have been funded through the U01 mechanism for preclinical pharmacology or compounds have been licensed to commercial companies in preparation for phase I clinical trials.

The organization of our drug discovery efforts and examples of a successful compound HTS experiment and a successful functional genomic screen relevant to cancer will be discussed.

Keywords: drug discovery, high-throughput screening, functional genomics

416 The Use of Genetically Engineered Mice to Investigate Treatment Response and Resistance

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The Mouse Models of Human Cancer Consortium (MMHCC) has the overall goal of generating accurate murine models and using these strains for biologic and preclinical research to advance our current understanding and treatment of human cancers. One focus of our MMHCC award is to engineer and interrogate models of hematologic malignancies characterized by aberrant Ras signaling. Somatic *RAS* mutations are the most common oncogenic alteration detected in human cancers. Ras proteins normally regulate cell fates by cycling between active GTP-bound and inactive GDP-bound states (Ras•GTP and Ras•GDP). Ras•GTP modulates cell fates by activating effector pathways that include the Raf/MEK/ERK, phosphoinositol 3'-kinase (PI3K)/Akt, and Ral•GDS cascades. Signaling terminates when Ras•GTP is hydrolyzed to Ras•GDP. GTPase activating proteins (GAPs) are negative regulators of Ras output that increase the rate of GTP hydrolysis. Mammalian cells express two major GAPs – p120GAP and neurofibromin. The latter is encoded by the *NF1* tumor suppressor gene, which is mutated in persons with neurofibromatosis type 1 (NF1). Children with NF1 are predisposed to juvenile myelomonocytic leukemia (JMML) and other cancers. Strains of mice carrying conditional mutant alleles of *Nf1,* oncogenic *Kras,* and oncogenic *Nras* are novel reagents for understanding how cells remodel signaling networks in response to hyperactive Ras and for performing preclinical trials. Use of the *Mx1-Cre* transgene to ablate *Nf1* or to activate oncogenic $Kras^{G12D}$ or $Nras^{G12D}$ expression in hematopoietic cells causes myeloproliferative disorders (MPDs) that model JMML and chronic myelomonocytic leukemia (CMML). We are using retroviral insertional mutagenesis (RIM) to identify cooperating mutations that might induce progression from MPD to acute myeloid leukemia (AML). CI-1040, a potent inhibitor of MEK, unexpectedly had no beneficial effects in *Nf1* mutant mice with MPD. By contrast, MEK inhibition induced regression of *Nf1*-deficient AMLs. These AMLs uniformly developed resistance *in vivo,* despite equivalent biochemical inhibition of the target in paired sensitive and resistant clones. Analysis of retroviral insertions in resistant AMLs revealed outgrowth of a pre-existing clone during CI-1040 administration, and we have implicated RasGRP1 and p38α as modulating resistance *in vivo* by cloning retroviral integrations from resistant leukemias. These data emphasize the importance of cell context in the response to targeted agents, and establish a tractable *in vivo* system for identifying genes that modulate therapeutic efficacy, and for uncovering mechanisms of acquired resistance that are likely to be relevant in human cancer.

Keywords: myeloid leukemia, RAS, therapeutics

417 A Phase II Study of Oral Mammalian Target of Rapamycin (mTOR) Inhibitor, RAD001 (Everolimus), in Patients With Recurrent Endometrial Carcinoma (EC)

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Background: PTEN mutations are present in 40-60% of EC, and the PTEN/PI3K/MTOR is a key pathway in the pathogenesis of EC. Loss of PTEN leads to constitutive activation of AKT, which leads to up-regulation of mTOR. RAD001 is an oral rapamycin analog that acts by selectively inhibiting mTOR.

Methods: A single institution, open-labeled, single arm, Phase II study in patients with recurrent EC who have failed at least one and no more than 2 prior chemotherapeutic regimens was performed. All patients had measurable disease. RAD001 was administered at a dose of 10 mg PO daily for 28 day cycles. One dose reduction (to 5 mg) was permitted. Patients were treated until progression or toxicity. The primary efficacy endpoint is Clinical Benefit Response (CBR), defined as a complete or partial response or prolonged stable disease (SD;>8 weeks) by RECIST criteria. Inclusion was limited to patients with endometrioid EC. Correlative studies evaluating PTEN were performed in primary hysterectomy specimens.

Results: Thirty-five patients were enrolled (median age 58; range: 38-81). A total of 81 cycles have been administered. 12 of 28 (44%) evaluable patients for response had CBRs. All of these patients had SD (median: 4 cycles; range 2-10). One patient achieving CBR is still on treatment. Patients with CBR discontinued treatment because of toxicity(6), progression(4), and noncompliance(1). 16 of the 28 patients discontinued treatment after receiving ≤2 cycles because of progressive disease (15) and toxicity (1). Seven patients were inevaluable after receiving ≤1 cycle because of toxicity (5) or clinical deterioration (2). The most common adverse events were abdominal pain (28%), nausea/vomiting (21%), low grade fever (21%), and anemia (17%). 17 patients required a dose reduction. Fifteen of 28 evaluable patients had tissue available from the original hysterectomy. Loss of PTEN expression was predictive of CBR with a sensitivity of 88%, specificity of 57%, positive predictive value of 70% and negative predictive value of 80% (P=0.10).

Conclusion: RAD001 shows encouraging single agent clinical benefit in pretreated patients with recurrent EC. Loss of PTEN may predict CBR. Future studies will evaluate this agent in combination with hormonal and/or cytotoxic therapy.

Keywords: endometrial cancer, mTOR inhibitor, RAD001

418 Using Theranostic Targets for the Identification and Elimination of Non-Invasive Basal-like Breast Carcinomas in Young Women

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Basal-like breast carcinomas account for ~15-20% of breast cancers in Caucasian women and are associated with poor clinical outcome. Currently, no optimized therapeutic agents have been identified for the treatment of this malignant disease. Additionally, this tumor subtype is more prevalent in young African American women and can represent up to 39% of diagnosed tumors. There is an urgent need for early detection and prevention strategies for this disease.

Previous studies have characterized the histologic and immunophenotypic properties of breast basal-like carcinomas that were first positively identified using DNA microarray analysis. The infrequent expression of myoepithelial markers in basal-like carcinomas does not support a direct myoepithelial cell derivation of these tumors. Using a biological analysis, we have recently demonstrated that a molecular hallmark of basal-like carcinomas is abrogation of the pRB tumor suppressor pathway, via pRb inactivation (Gauthier et al, Cancer Cell 12, 479-91, 2007). In a large retrospective study, we found that expression of biomarkers indicative of pRb inactivation and an abrogated response to cellular stress identified women diagnosed with DCIS that subsequently developed basal-like breast carcinomas. Thus, assessment of these biomarkers in DCIS has begun to allow prediction of basal-like tumor formation years before it actually occurs. Furthermore, our studies into the pathways involved in the origin of basal-like phenotypes have also allowed us to identify key steps in epigenetic modifications that allow the acquisition of the invasive transition of basal-like carcinomas. These steps provide a unique opportunity to target this subtype of breast carcinoma and eliminate the cells at risk prior to invasive carcinoma formation. Such intervention agents would be offered to patients where basal-like non-invasive carcinomas have been identified. In preparation for treating early disease, we have been characterizing HDAC (histone deacetylase) proteins as therapeutic targets. Current studies have been extending clinical trials to additional epigenetic modulators. In aggregate, our team has experience in identifying women at high risk for developing basal-like breast carcinomas before they form and experience in designing interdisciplinary, individualized care tailored to each person's risk factors. In summary, our team is continuing our development of a clinical tool that will stratify a woman's individual risk for developing a future basal-like invasive tumor after a diagnosis of DCIS and coupling this prognostic signature with an intervention that will eliminate the cells at risk for developing a malignancy in a concurrent fashion. Our in vitro and in vivo work credentials the concept and the targets and positions us for Phase I/II trials. Two steps of the Agents pathway are addressed.

Keywords: theranostic, prevention, basal-like carcinoma

419 Translational Research Program for Novel First-in-Human Cancer Drugs

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Background: In the current anticancer drug development era, the identification and validation of biomarkers that can: 1) determine which patients are most likely to benefit from specific targeted therapies (*predictive* markers) and 2) confirm biological mechanisms of action (*pharmacodynamic* markers), are of high priority and continue to pose challenges to clinicians and scientists. Most anticancer drugs are developed pre-clinically based on limited cell line models, and tumor sensitivities to drugs are divergent and potentially contradictory in cell lines growing on plastic compared to tumor mass in animals. A novel approach addressing this is to develop primary xenograft models directly from patients' tumors, recapitulating the properties of malignant growth in patients. Evaluation of these models provides the proof of mechanisms of drug activities and new insights into biomarkers of drug response.

Proposal: This proposal aims to establish a translational research program for ex-vivo ("outside-the-patient") evaluation of novel targeted anticancer drugs, using primary tumor xenografts established from fresh research biopsies of patients undergoing first-in-human Phase I studies of these agents. Through key linkages with informatics specialists, molecular data obtained are subjected to integrated computational analysis incorporating existing protein-protein interaction databases, pathway databases, external annotation databases, specialized resources and platforms linking clinical and therapeutic outcome data. In addition, using state-of-the-art imaging facilities and equipments, functional imaging of xenograft bearing animals can be evaluated for early read-out of antitumor activity. Lastly, the successful development of xenograft models depends on having a specialized Drug Development Team that can conduct intense clinical and drug level monitoring and drug activity analyses required in Phase I trials.

Research plan: In this program, a large number (n=100-200) of primary tumor xenograft models representing several common human cancers are established in mice using tumor biopsies of patients during first-in-man studies of novel anticancer drugs. These tumors are systematically characterized for their aberrant genomic and gene expression profiles, protein expression, genetic polymorphisms, as well as pharmacokinetic, pharmacodynamic and therapeutic responses in "inside-" and "outside-the-patient" studies of the novel drugs.

Application: The goal of this proposal is to define molecular pathways, aberrations or signatures that are highly associated with responses to specific drugs, thereby establishing biomarkers and paradigms for the selection of patients for targeted therapies. Such paradigms can then be used to refine early phase clinical trials for greater success rates, ultimately leading to a tangible goal of personalizing cancer care.

Keywords: tumor xenografts, biomarkers, Phase I trials

420 CEACAM6: A Promising Biomarker and Therapeutic Target for Pancreatic Adenocarcinoma

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Pancreatic cancer is the 5th leading cause of cancer-related death in the United States. Most patients present with surgically incurable disease, and currently available chemotherapeutic agents have only a modest effect on survival. As such, there is a need for new therapeutic and diagnostic strategies.

Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 6 is a glycosylphosphatidylinositol (GPI)-linked immunoglobulin superfamily member that functions as an intercellular adhesion molecule. Pancreatic adenocarcinoma cells differentially express CEACAM6. We hypothesized that CEACAM6 signaling contributes to the pathogenesis of pancreatic adenocarcinoma.

CEACAM6 overexpression was induced in Capan2 human pancreatic cancer cells, which inherently express low levels of CEACAM6. RNA interference was used to suppress CEACAM6 expression in BxPC3 human pancreatic cancer cells, which inherently express high levels of CEACAM6. CEACAM6 silencing was associated with increased anoikis susceptibility, decreased invasive potential, and decreased chemoresistance to gemcitabine. In contrast, forced CEACAM6 overexpression was associated with decreased anoikis susceptibility, increased invasive potential, and increased chemoresistance to gemcitabine.

In preclinical studies, mice implanted with pancreatic cancer xenografts were administered CEACAM6-specific siRNA, control siRNA, or PBS twice weekly by tail vein injection. Administration of CEACAM6-specific siRNA was associated with inhibition of tumor growth, prevention of metastasis (whereas 60% of PBS-treated mice and 50% of control siRNA-treated mice developed liver metastasis), and prolonged survival.

Finally, immunohistochemical analysis of CEACAM6 expression in pancreatic intraepithelial neoplasia (PanIN) lesions and in pancreatic adenocarcinoma specimens derived from patients having undergone resection for pancreatic cancer at our institution was performed. Tumoral expression levels of CEACAM6 were correlated with clinicopathological data from these patients. Patients with absent tumoral CEACAM6 expression had significantly longer overall postoperative survival (median 8.8 years) than those with CEACAM6-positive tumors (median 1.3 years; $P = 0.047$). Additionally, CEACAM6 expression was significantly overrepresented in patients with lymph node metastases (N1 versus N0) and in those higher T stage (T3 versus T2). Relative to low grade PanIN lesions (IA and IB), higher grade PanIN lesions (2 and 3) demonstrated significantly greater CEACAM6 expression ($P < 0.0001$).

In summary, CEACAM6 shows promise as both a biomarker and a therapeutic target in pancreatic cancer.

Keywords: CEACAM6, pancreatic cancer, adenocarcinoma

421 Monoclonal Antibody to Transforming Growth Factor β Inhibits Cancer Progression in Multiple Preclinical Cancer Models

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Transforming Growth Factor β (TGFβ) can have opposing functions in tumor progression, acting as a tumor suppressor in preneoplastic lesions and a tumor promoter once the disease has progressed. The Vanderbilt University Tumor Microenvironment Network compared the use of neutralizing antibodies to TGFβ as a therapeutic approach in breast and prostate cancer and breast metastasis to bone.

Spontaneous Transgenic Breast Cancer: In the Polyoma Virus Middle T (PyVmT) mouse model of breast cancer, the animals start to develop hyperplastic lesions in all mammary glands at 4 to 6 weeks of age. By 8 weeks of age the lesions become invasive and lung metastases develop. PyVmT mice were treated with the TGFβ neutralizing antibody 2G7 (Biswas et al. JCI, 2007) at 15 ug/gm body weight twice every week either from 4 to 13 weeks or 8 to 13 weeks of age. Primary tumor burden was measured using PET/CT imaging technique. Tumor burden was decreased compared to untreated controls when the mice were treated from 8 weeks to 13 weeks of age. However, treatment from 4 weeks of age increased both tumor burden and lung metastasis. These results suggest that anti-TGFβ strategy might only be useful in treating advanced-stage breast cancer and can be detrimental to early-stage breast cancer.

Prostate Cancer: Human prostatic carcinoma-associated fibroblasts (CAF) induce tumorigenesis in initiated but non-malignant human prostatic epithelial cells (BPH-1). CAFs express elevated levels of TGFβ1and stromal cell derived factor (SDF-1/CXCL12). TGFβ inhibits the growth of BPH-1 cells *in vitro*, however, was found to be necessary for the tumorigenic response to CAF. BPH1+ CAF recombinants were grafted under the kidney capsule of mice preloaded with an anti-TGFβ antibody 2G7 (15 ug/gm body weight). In contrast to the *in vitro* results overall tumor volume was significantly reduced by TGFβ inhibition and invasive malignant histology ablated in the treatment group *in vivo*. Our data suggested that the mechanism was via TGFβ upregulation of the chemokine receptor CXCR4 on BPH-1 cells, which activates Akt in response to the CAF-produced ligand SDF1. These experiments suggest a mechanism by which TGFβ can shift from a growth inhibitor to a tumor promoter during tumor progression and suggests that TGFβ inhibition may be effective in early-stage prostatic carcinogenesis (Ao et al, Can Res, 2007)

Breast Cancer Bone Metastasis: We tested the effect of systemic administration of a pan-TGFβ antibody 1D11(Genzyme corporation) in breast-to-bone metastasis. MDA-MB-231 human breast cancer cells were injected in 4-5 weeks old female nude mice via the left cardiac ventricle. Tumor- bearing mice were treated with either 1D11 or control antibody (13C4) intraperitoneally three times every week for two weeks. Treatment was initiated two weeks after tumor inoculation. Efficacy of 1D11 on metastatic progression of breast cancer cells in bone was tested using faxitron analysis of bone lesions, microCT analysis on bone volume and histomorphometry to examine tumor burden in bone. Treatment with 1D11(10ug/g, three times a week for two weeks) reduced the number of bone lesions (Control: 7.4, Treated: 2.3, $P<.005$), and area of bone lesions (control: 7535.5 cm2, treated: 865.5 cm2, $P<.001$) and increased tibial bone volume (Control: 0.093, Treatment: 0.198, $P=.01$). In this preclinical model, anti-TGFβ antibody effectively reduced number and area of metastatic bone lesions.

We conclude that inhibition of TGFβ signaling using neutralizing antibodies can be effective treatment for late-stage breast cancer metastasis to lung and bone, but is complicated by opposing effects on earlier-stage breast cancer. The inhibitory effect of anti-TGFβ on CAF induced prostate cancer suggests that this strategy may also be useful in prostate cancer.

Keywords: TGF-beta, breast cancer, prostate cancer

422 Combined Modality Targeted Therapy of Pancreatic Cancer With Death Receptor Monoclonal Antibodies

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There is accumulating evidence that targeting death receptors on the surface of cancer cells with their native ligand, TRAIL, or death receptor-specific agonistic MAbs represents a promising anti-cancer therapeutic approach. DR5 death receptor expression in pancreatic carcinoma cell lines and effects of TRA-8 (anti-DR5) MAb and chemotherapy treatment were evaluated *in vitro*. Baseline cell surface expression of DR5 was high in MIA PaCa-2 and S2-VP10 cells, and increased after 48 h treatment with Gemcitabine. MIA PaCa-2 and S2-VP10 cells were selected as representative of TRA-8 sensitive and resistant cell lines, respectively, for combination cytotoxicity studies with Gemcitabine. MIA PaCa-2 cells were moderately resistant to Gemcitabine, which produced additive cytotoxicity when combined with TRA-8. S2-VP10 cells were sensitive to Gemcitabine, and treatment with Gemcitabine produced synergistic cytotoxicity when combined with TRA-8.

We explored the anti-tumor effects of TRA-8 alone, and in combination with CPT-11, or Gemcitabine in an orthotopic pancreatic cancer xenograft model. SCID mice with MIA PaCa-2 intrapancreatic tumors were treated with TRA-8, Gemcitabine, or combination therapy. Both TRA-8 and combination treatment resulted in significant tumor growth inhibition compared to the untreated group or the Gemcitabine treated group. Mean survival times were 76 ± 3, 79 ± 5, 121 ± 4, and 142 ± 7 days in the untreated, Gemcitabine, TRA-8, and TRA-8 + Gemcitabine treatment groups, respectively. We then examined the efficacy of two treatment cycles with TRA-8 and CPT-11. There was significant enhancement of survival for single agent TRA-8 or CPT-11 compared to untreated animals while combination therapy animals had mean survival of 169.2 ± 4.2 days which was significantly longer than the other 3 groups.

We have completed a Phase I, single center dose escalation trial to determine safety and maximal tolerated dose (MTD) of humanized TRA-8 (CS-1008) given intravenously (IV) as a single agent in patients with relapsed/refractory solid tumors. The protocol was a dose escalation strategy with 4 cohorts in a dose range from 1-8 mg/kg weekly X 6. Seventeen patients were enrolled (7 males, 10 females) with a median age of 57 years (range 31-88 years). Nine patients were enrolled in the I, 2, and 4 mg/kg dose cohorts (3 in each) and 8 patients in the 8 mg/kg dose cohort. CS-1008 was well tolerated with no DLTs observed, and the MTD was not reached. There were no infusion related toxicities and no drug-related grade 3 or 4 toxicities. Pharmacokinetic results demonstrate a blood half-life of 8 to 16 days with approximately 3-fold accumulation of the antibody at steady state. No anti-CS-1008 antibodies were detected. Seven patients had stable disease: 2 in the 1 mg/kg dose cohort (hepatocellular carcinoma 497+ days and head and neck cancer 225 days), 1 in the 2 mg/kg cohort (colon cancer 77 days), 2 in the 4 mg/kg dose cohort (colon cancer 78 days and cholangiocarcinoma 155 days), and 2 in the 8 mg/kg dose cohort (colon cancer 79 days and hepatocellular carcinoma 78 days). The high number of patients with stable disease in this Phase I trial suggests anti-tumor activity.

A Phase II trial of hTRA-8 (CS-1008) plus Gemcitabine in previously untreated patients with metastatic pancreatic cancer is ongoing. This is a multi-institutional trial led by the UAB SPORE in Pancreatic Cancer and includes 9 other institutions.

Keywords: pancreatic cancer, death receptor antibody, chemotherapy

423 Expression Profiling Reveals That Epithelial-to-Mesenchymal Transition (EMT) Controls Global Drug Resistance in Pancreatic Cancer

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Pancreatic cancer remains a devastating disease for which no effective treatment options exist. Gemcitabine-based regimens are the current standard-of-care but there is emerging consensus that they are primarily palliative and do not substantially alter the course of disease progression (1). We characterized the effects of four conventional chemotherapeutic agents with divergent mechanisms of action (gemcitabine, 5-fluorouracil, taxol and cisplatin) on apoptosis in a panel of 11 common human pancreatic cancer cell lines. The results demonstrated that five (HPDE, L3.6pl, BxPC3, CFPAC, Su8686) were sensitive to all four agents and six (Panc-1, HS766T, ASPC-1, MiaPaca-2, L3.6plGR, MPanc96) were resistant to all 4 agents as measured by MTT reduction and propidium iodide-FACS-based quantification of apoptosis-associated DNA fragmentation. In an attempt to identify the mechanisms involved in apoptosis resistance, we performed whole genome expression profiling using Illumina microarrays. Unsupervised hierarchical clustering revealed that the sensitive and resistant cells formed two distinct groups that were distinguished by markers of "epithelial-to-mesenchymal transition" (EMT). Interestingly, an inverse correlation between E-cadherin (epithelial marker) and one of its transcriptional suppressors (Zeb-1) (2) was observed in the gene expression data and confirmed by real-time PCR. Silencing of Zeb-1 in the mesenchymal lines increased not only the expression of E-cadherin, but also other epithelial markers such as EVA1 and MAL2 and restored gemcitabine sensitivity. Importantly, immunohistochemical analysis of E-cadherin and Zeb-1 in primary tumors confirmed that expression of these two proteins was mutually exclusive and that the majority of tumors (2/3) possessed a "mesenchymal" phenotype.

Zeb-1 functions by recruiting histone deacetylases (HDACs) to E-box elements located within E-cadherin's promoter (2). Consistent with other emerging data (3), two different HDAC inhibitors (SAHA and SNDX-275) increased E-cadherin expression and decreased Zeb-1 expression in vitro and in orthotopic mPanc-96 xenografts in vivo. Because HDAC inhibitors promote accumulation of p21 and G_1 arrest (4), we wondered whether the effects of the combination would be improved by removing the HDAC inhibitor prior to adding an S-phase active agent like gemcitabine. Preincubation with SAHA or SNDX-275 for 24 h followed by drug washout resulted in strong resensitization of Panc-1 cells to gemcitabine. Together, these results demonstrate that HDAC inhibitors can be used to reverse EMT and drug resistance in preclinical models in vitro and in vivo. We are planning a Phase I/II clinical trial of SNDX-275 plus gemcitabine to determine whether these preclinical observations can be exploited in pancreatic cancer patients. Because cancer stem cells also appear to display features of EMT (5), HDAC inhibitors may help to reverse drug resistance in the cancer stem cell compartment as well.

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Keywords: gemcitabine, apoptosis, Zeb-1/deltaEF1/TCF8, SAHA, SNDX-275

424 Host Polymorphisms in the IL6 Promoter Predict Poor Outcome in Patients With ER-Positive, Node-Positive Breast Cancer

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Interleukin-6 (IL-6) modulates immune response, estrogen production and growth pathways in breast cancer. Common polymorphisms in the *IL-6* promoter regulate transcription. We previously reported an association between the IL6-174GG genotype and decreased disease-free and overall survival among women with ER-positive, node-positive breast cancer[1]. We sought to further evaluate the full complement of functional variants in the IL6 promoter (SNPs -572G>C, -597G>A and repeat -373 A_nT_n) in node-positive patients enrolled on a multicenter, cooperative group trial of adjuvant chemotherapy for breast cancer.

Genomic DNA and clinical data from patients enrolled on E2190/Intergroup 0121 were collected. Genotyping for -597G>A, -572G>C, -174G>C and -373AnTn was performed by PCR using site-specific primers and PyroSequencing and sequencing for the repeat polymorphism. Log-rank tests and Cox modeling were used to compare outcomes by genotype/haplotype and other factors. Stratification on estrogen receptor status was prespecified.

344 patients (64% of parent trial) had corresponding genotype/clinical data available and did not differ from overall trial participants. After adjustment for other prognostic factors, those patients with ER positive tumors and genotypes 597GG or 174GG had significantly worse DFS (HR 1.6, p=0.02 and HR 1.71, p=0.007, respectively), while the 373 8A12T repeat appeared to be protective (HR 0.62, p=0.02). The G-G-[10A/11T]-G haplotype was associated with worse DFS (HR 1.46, p=0.04), while the A-G-[8A/12T]-C haplotype was associated with improved DFS (HR 0.69, p=0.009).

Functional polymorphisms in *IL-6* identify an ER-positive population with poor outcome. These findings support mechanistic studies aimed at development of host-targeted approaches to improve treatment for ER-positive breast cancer.

Keywords: breast cancer, polymorphism, interleukin-6

425 Improved Synthesis and Antitumor Efficacy of 6-*epi*-Dictyostatin in MDA-MB231 Human Breast Cancer Xenograft-Bearing Mice

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Structure-activity studies centered on the naturally occuring microtubule stabilizing agent dictyostatin have recently identified several highly active epimers and analogs. From these compounds, 6-*epi*-dictyostatin was selected for scale-up preparation and evaluation in animals. Here we describe a new total synthesis that produced more than 30 mg of 6-*epi*-dictyostatin. The compound was found to have potent antitumor activity in SCID mice bearing MDA-MB231 human breast cancer xenografts. 6-*epi*-dictyostatin was more effective than paclitaxel when administered intravenously in three doses of 20 mg/kg/dose spaced 7 days apart to C.B-17 SCID female mice bearing established MDA-MB231 human breast cancer xenografts. Mean tumor volumes in the 6-*epi*-dictyostatin-treated mice were significantly smaller than those in the control and vehicle-treated groups beginning on day 7 of treatment, and smaller than those in the paclitaxel-treated group beginning on day 10 of treatment. Tumor regression was observed in six of the 6-*epi*-dictyostatin-treated mice on day 14, and the tumors did not grown in these mice. On day 28, tumor regrowth was observed in the remaining three 6-*epi*-dictyostatin-treated mice. Tumor continued to grow in all the paclitaxel-treated mice, albeit at a slower rate than that observed for the tumors in the control and vehicle-treated groups. Body weight loss was less than 10% in the 6-*epi*-dictyostatin-treated mice. Tumors in the 6-*epi*-dictyostatin-treated mice did not double in volume at 28 days. The mean tumor doubling times for the paclitaxel-treated mice were significantly longer than the tumors in the control and vehicle-treated groups. Both the median optimal %T/C (day 14) and median optimal %T/V (day 17) were approximately 30% for the paclitaxel-treated mice, and 13% for the 6-*epi*-dictyostatin-treated mice, a significant difference. Studies are ongoing to determine tissue distribution, metabolism, and tumor vs. non-target tissue pharmacodynamic effects.

Keyword: microtubule, natural product, xenograft

426 Effect of Exemestane on Bone Mineral Density in Postmenopausal Women at Risk for Breast Cancer: Preliminary Analysis

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Background: The aromatase inhibitors (AIs) show promise for breast cancer prevention in postmenopausal women. However these agents have been shown to decrease bone mineral density in women with breast cancer. Exemestane, a steroidal aromatase inhibitor, may be more bone sparing than the non-steroidal aromatase inhibitors.

Methods: We conducted a phase II trial of exemestane in postmenopausal women at increased risk of breast cancer to assess the effect on anterior-posterior (AP) spine and total hip bone mineral density (BMD). Eligible participants are at increased risk for breast cancer by virtue of: Gail model risk $\geq$ 1.7% over 5 years; high risk pathological lesion (e.g. lobular neoplasia, ductal carcinoma in situ); known *BRCA1/2* deleterious mutation or prior stage I/II breast cancer at least 2 years from breast cancer treatment and not treated with AIs. Women were required to undergo DEXA scan of the AP spine and hip at baseline and were excluded if AP spine T-score was $\leq$-2.5. AP spine measurements were done twice at each time point and the results averaged. Study participants receive exemestane 25 mg, calcium carbonate 1200 mg and vitamin D 400 IU daily for two years. We report the results of a planned interim safety analysis assessing one year change in bone density in the first 18 subjects. Student's t-test was used to determine relative change from baseline in AP spine and total hip BMD.

Results: To date, 30 women have enrolled in the trial and 21 have completed 1 year of exemestane. Three women discontinued agent after an average of 3 months due to joint pain. For the 21 included in this analysis 15 were eligible due to a high risk pathological lesion, 4 by Gail Model and 2 by prior breast cancers. The average change in AP spine BMD from baseline to 1 year (N= 21) is –1.3 %, range -10.0 to +7.0% (p=0.20 for the null hypothesis of zero overall change; 95% C.I. for the mean -3.2% to +0.7%). Average AP spine T score is -0.28 at baseline and -0.45 at one year. Change in total hip BMD is -1.4%, range -7.5% to 5.9% (p=0.058, 95% C.I. -2.8% to +0.1%). Average total hip T score is -0.05 at baseline and -0.17 at one year.

Conclusions: These preliminary data suggest that exemestane 25 mg/day for one year with concurrent calcium and vitamin D has acceptable effects on bone density in postmenopausal high risk women, although there were wide ranges in effects. The observed average reduction in BMD is less than the reported effect in treatment studies of women with invasive breast cancer. Studies are in progress to determine whether exemestane use by postmenopausal women is effective in reducing the risk of invasive breast cancer.

Keywords: exemestane, prevention, biomarkers

427 Endoxifen, but not 4-hydroxytamoxifen (4HT), Degrades the Estrogen Receptor (ER) in Breast Cancer Cells: An Impetus for Endoxifen Drug Development

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Background: Tamoxifen (Tam) is activated by the cytochrome P450 2D6 enzyme system into two potent and active metabolites, 4-hydroxytamoxifen (4HT) and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). Patients (pts) with genetically impaired CYP2D6 metabolism have significantly reduced endoxifen concentrations and a higher risk of recurrence (Goetz et al *J Clin Oncol* 2005 and Goetz et al *Breast Cancer Res Treat* 2007). These data led an FDA subcommittee to recommend a tamoxifen label change. We have gone on to perform comprehensive *CYP2D6* genotyping (*3, *4, *6, *10, *17, and *41) in the randomized NCCTG 89-30-52 adjuvant Tam trial and demonstrated that compared to CYP2D6 extensive metabolizers (EM), poor metabolizers (PM) had significantly shorter time to recurrence (HR 4.0, p=0.001) and DFS (HR 2.0, p=0.02) (Goetz/Ames/Ingle). Despite these observations, endoxifen's contribution to tam's overall drug effectiveness continues to be uncertain since the assumption is that endoxifen and 4HT have a similar mechanism of action on the ER

Methods: Using cells endogenously expressing ERα (MCF7, T47D) and cells stably transfected with ERα (Hs578T and U2OS), we examined the relative effects of Tam and its primary metabolites on ERα protein levels by western blotting, ERα transcriptional activity by luciferase reporter assays, and on ER positive breast cancer cell growth through the use of proliferation assays (Wu submitted).

Results: Endoxifen induces ERα protein turnover through proteasomal degradation similar to that of ICI in a concentration and time-dependent manner. These findings are in stark contrast to Tam, N-desmethyl-tamoxifen (NDT) and 4HT, which stabilize the ER. Optimal degradation occurs only at endoxifen concentrations observed in human CYP2D6 EM (> 40 nM) and persists even in the presence of concentrations of Tam (300 nM), 4HT (7 nM), and NDT (700 nM) observed in patients receiving Tam therapy. In contrast, reducing endoxifen concentrations to those observed in a CYP2D6 PM (20 nM), without altering Tam, 4HT, and NDT, results in ER stabilization. High endoxifen concentrations (100-1000 nM) completely block estrogen (E_2)-induced ER transcriptional activity even in the presence of Tam, 4HT, and NDT, while low endoxifen concentrations (20-40 nM) do not. Further, low concentrations of endoxifen (20 nM) do not significantly alter E_2-induced cell proliferation; however, high concentrations of endoxifen (100-1000 nM) completely block this process. Endoxifen pharmacokinetic studies are ongoing in mice to determine the oral bioavailability of endoxifen (Ames/Reid)

Conclusion: Endoxifen is a potent anti-estrogen that targets ERα for proteasomal degradation, blocks ERα transcriptional activity and inhibits E_2-induced breast cancer cell proliferation. These data suggest that Tam's optimal drug effect in breast cancer cells may be related to the ability of its metabolite, endoxifen, to degrade rather than stabilize the ER. Further work is necessary to determine whether endoxifen can be orally administered for the endocrine therapy of ER positive breast cancer.

Keywords: endoxifen, tamoxifen, CYP2D6

428 Genomic Pathways Approach to Biomarker Discovery for the Early Detection and Risk Assessment of Pancreatic Cancer

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In association with the NCI Early Detection Research Network, we are taking a targeted genomic approach to identify biomarkers for the early detection of pancreatic cancer. We hypothesize that genetic pathways involving tumor suppressor genes with loss of function mutations and/or deletions and dominantly activated, amplified, and/or overexpressed oncogenes are critical determinants in the development of early phases of pancreatic neoplasia. We furthermore hypothesize that by targeting specific pathways involving loss of chromosome 3p and 1p and gain of chromosome 12p and 20q, we will identify biomarkers for the early detection of pancreatic cancer.

Using functional complementation assays, we previously identified a chromosome 3p12 tumor suppressor locus proximal to the most common fragile site in the human genome and within a genomic interval identified as initiating event in a cytogenetic pathway associated with smoking related tumors. In order to identify a chromosome 3p pathway in pancreatic cancer, 880 partial cDNAs were obtained from a suppression subtractive hybridization library (SSH) constructed to identify chromosome 3p12 pathway genes. cDNAs differentially expressed from the SSH library were compared to expression profiles from an Affymetrix GeneChip array interrogated with RNAs derived from the same starting materials used for SSH library construction to identify a subset of genes across these two platforms that were differentially expressed. Affymetrix arrays were then interrogated with tumor/normal pancreatic samples to further stratify genes based on a third expression platform. Results have identified 8 genes that are consistently differentially expressed across the three expression platforms and verified by quantitative RT-PCR. Two/eight genes (KSF-1 and KSF-2) have been shown to be upregulated and secreted in pancreatic cancers. Preliminary screening of 80 plasma samples from pancreatic cancer patients has identified KSF-1 as being both secreted and significantly differentially expressed. In addition, one of the three genes identified (KSF-3) is expressed abundantly only in the normal pancreas and downregulated in pancreatic cancers. A panel of markers is being assembled for prevalidation studies.

In addition, we performed molecular profiling of both gene expression and genomic copy number on *in vitro* pancreatic cancer cell lines as well as tumor samples using multiple microarray platforms. This cross validation approach in the *in vitro* and *in vivo* samples identified common candidate genes that were examined using pathway analysis software for interactions among multiple pathways and networks. An independent gene expression data set on normal pancreas, chronic pancreatitis and adenocarcinoma was then used to analyze the profiles of the candidate genes and pathways identified. Those profiles which were common to pancreatitis and pancreatic cancer as well as those unique to either were examined to specify the pathways potentially involved in the development of malignancy in an inflammatory background that is typical of pancreatic cancer.

Candidate biomarkers are also being examined for risk assessment in pancreatic cancer using single nucleotide polymorphism analysis. The overall goal is to develop a panel of biomarkers for the early detection and risk assessment of pancreatic cancer.

Keywords: pancreatic cancer, genomic pathways, biomarker discovery, early detection

429 Pancreatic Cancer Radiosensitization by Nelfinavir

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Purpose: Both local and systemic failures can be life-threatening for patients with adenocarcinoma of the pancreas. Nearly all pancreatic cancers harbor activating mutations in the K-*ras* oncogene. We have previously shown that aberrant activation of the P-I3K/Akt signaling pathway, located downstream of K-*ras*, contributes to radioresistance in pancreatic cancers. FDA-approved agents capable of blocking Akt activity include the HIV protease inhibitor nelfinavir. We hypothesized that inhibition of Akt by nelfinavir would render pancreatic cancer cells more sensitive to ionizing radiation.

Methods: Several well-established pancreatic cancer cell lines (chosen based on their ability to be used in clonogenic survival assays) were obtained from the ATCC and maintained according to recommendations. Inhibition of Akt activation was assessed by western blot of phospho-Akt (Cell Signaling Technology) following treatment with nelfinavir (1 μM). The effect of nelfinavir (1 nM to 50 μM) on cellular proliferation was assessed by MTS assay (Promega). Cell cycle distribution was assessed via propidium-iodide staining and flow cytometry after treatment of exponential growth phase cells with nelfinavir (1 μM for 24 hours). Single cell suspensions were plated and incubated overnight to ensure log-phase growth prior to treatment with nelfinavir (1 μM) or control. Cells were irradiated with single doses of radiation after 24 hours of drug treatment. After 2 weeks, colonies were counted and clonogenic survival curves generated to assess *in vitro* radiosensitization. A cell line xenograft model was used to evaluate radiosensitization after oral dosing of nelfinavir.

Results: Nelfinavir treatment inhibits Akt, but not ERK, phosphorylation in a time and dose dependent manner after treatment of multiple cell lines. This occurs in both wild-type K-*ras* expressing (T3M4) and mutant K-*ras* expressing (MIAPaCa-2, Capan-2, and Panc-1) cell lines. Doses which inhibited Akt phosphorylation (1 μM) did not affect cell proliferation or cell cycle distribution in any cell line. At physiologically attainable doses, nelfinavir (1 μM) radiosensitized cell lines regardless of K-*ras* mutation status (enhancement ratios 1.37, 1.17, 1.22, and 1.32, respectively for T3M4, MIAPaCa-2, Capan-2, and Panc-1). Results from treatment of mouse xenografts will also be presented.

Conclusions: Nelfinavir, an FDA-approved HIV protease inhibitor, blocks activation of Akt and sensitizes pancreatic carcinoma cells to radiation. Further investigation is warranted into nelfinavir's radiosensitizing properties and its use in combination with chemoradiation for localized treatment of pancreatic cancer.

Keywords: radiation sensitization, protease inhibition, nelfinavir

430 Targeted BIKDD Expression Promotes the Therapeutic Efficacy of Lapatinib for Breast Cancer Treatment

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Anti-HER2 targeting therapies have been successfully applied in advanced breast cancer patients. The clinical efficacy of anti-HER2 agents is determined by their ability of promoting apoptosis. Loss of ability of inducing apoptosis will lead to no response or resistance following drug treatment. The rational combination therapy of anti-HER2 and apoptosis-promoting agents is urgently needed. Here, we describe a targeted approach to promote the therapeutic efficacy of lapatinib, which is a tyrosine kinase inhibitor to epidermal growth factor receptor and HER2/neu, in breast cancer treatment. We developed a potent breast cancer-targeting expression vector, driven by an engineered VISA-based claudin-4 promoter. Targeted expression of BIKDD, a potent proapoptotic gene driven by VISA-Claudin4 vector, demonstrated potent antitumor efficacy *in vitro* and *in vivo* without toxicity. Furthermore, it sensitized breast cancer cells to lapatinib treatment, and exhibited synergistic effects with lapatinib in several breast cancer cell lines *in vitro* and *in vivo*, without affecting the normal cell lines. Thus, a combination treatment of BIKDD and lapatinib will likely be beneficial to patients who have high EGFR or HER2 expression with BCL-2 or MCL-1 overexpression, or who experience tumor recurrence via the inhibition of apoptosis after lapatinib treatment.

Keywords: HER2; BikDD; Lapatinib

431 Loss of PTEN Engages ErbB3 and IGF-I Receptor Signaling to Promote Antiestrogen Resistance in Breast Cancer

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We sought to determine whether loss of the lipid phosphatase PTEN confers resistance to antiestrogens in estrogen receptor (ER)-positive breast cancer cells and primary tumors. Stable knockdown of PTEN expression (PTEN-kd) with shRNA in MCF-7, T47D, and MDA-361 ER+ human breast cancer cells resulted in upregulation of PI3K and P-Akt, resistance to tamoxifen and faslodex, and estrogen-independent growth. Upon PTEN loss, ER levels were maintained in MCF-7 cells but markedly reduced in T47D and MDA-361 cells. Increased PI3K signaling has been shown to prime estradiol-dependent and -independent, ER-mediated transcription. While PTEN loss upregulated basal and estradiol-stimulated ER transcriptional reporter activity in MCF-7 cells, opposite effects were seen in T47D and MDA-361 cells.

We then evaluated mechanisms of PI3K activation in PTEN-kd cells by immunoprecipitating p85, the PI3K regulatory subunit, and examining p85-bound tyrosine-phosphorylated adaptors or receptors. Loss of PTEN increased the association of p85 with IRS-1 (MCF-7) and with ErbB3 (T47D and MDA-361). PTEN-kd increased sensitivity to IGF-I in MCF-7 cells. T47D/PTEN-kd and MDA-361/PTEN-kd cells showed maximal PI3K activation under basal conditions, while PI3K remained inducible by serum in PTEN+ control cells. PTEN-kd cells had increased and prolonged activation of IGF-IR and ErbB3, thus implicating PTEN in the regulation of signaling upstream of PI3K. Further, PTEN loss increased non-genomic, estrogen-induced signaling via IGF-IR by increasing the association of p85 with IRS-1 and the subsequent activation of PI3K/Akt in MCF-7 cells. Inhibition of IGF-IR with the small molecule AEW541 and/or inhibition of HER2-mediated activation of ErbB3 with lapatinib restored the growth inhibitory effect of antiestrogens. PTEN-kd cell growth was suppressed by BEZ235, a small molecule inhibitor of PI3K and mTOR.

Using these cell lines, we generated a common 24-gene expression signature of PTEN loss by microarray analysis. Comparison of the gene expression signature in all 3 PTEN-kd cell lines to the Connectivity Map (*Science* 313:1929, 2006) showed that it was negatively associated with that induced by the PI3K inhibitors wortmannin and LY294002 (p=???), suggesting it reflected activation of PI3K. We next used this signature to score the gene expression profiles of tumors from a cohort of 268 patients with ER+ breast cancer treated with adjuvant tamoxifen for 5 yrs and a median follow-up of 9.1 years (*BMC Genomics* 9:239, 2008). Patients with tumors with a gene expression signature of PTEN loss exhibited shorter relapse-free survival (p<0.0001; log rank test). Eleven genes in the PTEN loss signature were individually predictive of disease outcome (p<0.05). In a separate smaller cohort of patients with ER+ cancers treated with adjuvant tamoxifen (n=34), undetectable PTEN in tumor cells as measured by immunohistochemistry also correlated with shorter relapse-free survival compared to tumors with detectable PTEN (p=0.06). These data suggest that 1) PTEN loss confers antiestrogen resistance to ER+ breast cancer by genomic and non-genomic mechanisms; 2) PTEN loss is permissive for activation of IGF-IR and ErbB3 signaling; 3) inhibition of the IGF-IR and/or ErbB signaling pathways overcomes the resistance to antiestrogens conferred by PTEN loss; and 4) a gene expression signature reflective of loss of PTEN and/or absence of PTEN protein can predict poor patient outcome after adjuvant hormonal therapy.

Keywords: antiestrogens, tumor suppressor, resistance

432 Effects of Methyl Derivatives of the Rexinoid UAB30 on Methylnitrosourea (MNU) Induced Mammary Cancers and on Various Indicators of Toxicity

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UAB30 is a RXR selective retinoid that has shown excellent activity in preventing chemically-induced mammary cancers with minimal toxicity (specifically not increasing serum triglycerides), and is currently undergoing a Phase I clinical trial. Several methyl analogs were synthesized in an attempt to obtain a more active preventive agent and to better understand the mechanisms of activity/toxicity of this class of agents. The retinoids included 4-methyl-UAB30, 5-methyl-UAB30, 6-methyl-UAB30, 7-methyl-UAB30, and 8-methyl-UAB30. The compounds were evaluated in the MNU-induced mammary cancer model using female Sprague-Dawley rats. MNU (75 mg/kg BW) was administered at 50 days of age and the animals observed for 120 days afterward for the development of ER+ mammary cancers. The retinoids were given at a dose of 200 mg/kg diet, except 7-methyl-UAB30 (given at 100 mg/kg diet). 4-Methyl-UAB30 and 7-methyl-UAB30 were highly active; reducing mammary cancer multiplicity by 74 and 61%, respectively. The retinoids 5-methyl-UAB30, 6-methyl-UAB30, and 8-methyl-UAB30 did not have chemopreventive activity in this model. The 8-methyl-UAB30 derivative actually caused a 108% increase in growth of the mammary cancers. As we have previously shown (Carcinogenesis 27, 1232-1239, 2006), serum triglycerides correlated with cancer preventive activity; i.e., high levels were observed with active retinoids. 4-methyl-UAB30 caused an initial large increase in body weight gain of the rats. Of interest, serum levels of 6-methyl-UAB30 and 7-methyl-UAB30 were high, while the other agents were low or could not be detected. All methyl derivatives caused varying decreases in liver retinyl palmitate levels. Structure-activity relationships are also being evaluated using crystallography of RXR/UAB-rexinoid complexes as a guide. Structure-based and dynamic-based approaches are used to facilitate the design of new rexinoids that fit into the LBD of RXRs. For example, 4-methyl-UAB30 had a 5-fold greater binding affinity to hRXR alpha LBD than 9-cis-retinoic-acid. These studies emphasize that minor modifications of a retinoid molecule can drastically change its absorption, metabolism, toxicity, binding affinities to receptors, and activity in preventing mammary cancers. (Supported by NCI Breast SPORE CA089019).

Keywords: breast cancer, retinoid, animal model

433 Effects of Biomechanical Properties of Bone on the Behavior of Breast Cancer Cells Resident in Bone

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Impaired quality of residual bone, as well as decreased bone amount, is common in patients with advanced breast cancer, and leads to skeletal complications that impair quality of life, such as bone pain and pathologic fracture. This is of increasing importance as patients live longer with metastatic disease, and is compounded by current therapies such as aromatase inhibitors. Our hypothesis is that whereas bone loss at the metastatic site is determined by osteoclast activity induced by the breast cancer cells, the quality of the residual bone at the tumor-bone interface is determined by osteoblast differentiation, which is frequently impaired in metastatic breast cancer. Furthermore, we propose that this is a consequence of ambient TGFβ concentrations which are enriched in the microenvironment of metastatic breast cancer cells in bone, and can be decreased by anti-TGFβ therapy, which we hypothesize will enhance osteoblast differentiation as well as improving bone quality. We are examining the elastic modulus of bone at the site of lytic metastases, at the tumor-bone interface, and at sites far removed from tumor in preclinical models. Modulus is being assessed by nanoindentation. We are manufacturing artificial substrates that mimic the biomechanical properties and determining the effects of these substrates on expression profiles in metastatic breast cancer cells.

We are investigating the specific role of TGFβ in osteoblast differentiation, bone structure and quality both in patients and preclinical models of breast cancer metastasis. Preclinical models will provide important information for the design of clinical studies. We are examining the effects of impaired TGFβ signaling in osteoblasts by the use of mice with conditional knockout of the TGFβ receptor kinase, and assessing bone quality as well as bone structure by state-of-the-art techniques including Raman spectroscopy, Atomic Force microscopy and μCT. We are also examining the effects of anti-TGFβ therapy using anti-TGFβ antibodies on bone quality, in parallel with its effects on tumor burden, osteoblast differentiation and bone structure in mice bearing human breast cancer cells, to guide the design of clinical studies, and provide information on the spectrum of benefits from anti-TGFβ therapy. We plan ultimately to evaluate the effects of anti-TGFβ antibodies on bone in a phase I study in patients with metastatic breast cancer. These studies focus on an important complication of breast cancer that markedly influences the quality of life in patients with advanced disease, and should have important therapeutic implications.

Keywords: bone metastasis, TGFβ, bone quality, breast cancer

434 Regulation of Pancreatic Tumor Cell Proliferation and Chemoresistance by the Histone Methyltransferase EZH2

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Purpose: Enhancer of zeste homologue 2 (EZH2), a histone methyltransferase, plays a key role in transcriptional repression through chromatin remodeling. Our objective was to determine the expression pattern of EZH2 and assess the anticancer effect of EZH2 depletion in pancreatic cancer cells.

Experimental Design: Immunohistochemistry and cytosolic/nuclear fractionation were performed to determine the expression pattern of EZH2 in normal pancreas and human pancreatic tumors. We used RNA interference, Western blotting, RT-PCR, and chromatin immunoprecipitation to study the effect of EZH2 depletion on pancreatic cancer cell proliferation and survival.

Results: We detected nuclear overexpression of EZH2 in pancreatic cancer cell lines and in 71 of 104 (68%) cases of human pancreatic adenocarcinomas. EZH2 nuclear accumulation was more frequent in poorly differentiated pancreatic adenocarcinomas (in 31 of 34 cases, $p<0.001$). We found that genetic depletion of EZH2 results in re-expression of $p27^{Kip1}$ through epigenetic regulation of the $p27^{Kip1}$ gene promoter and decreased pancreatic cancer cell proliferation. Moreover, we showed that EZH2 depletion sensitized pancreatic cancer cells to doxorubicin and gemcitabine leading to a significant induction of apoptosis suggesting that the combination of EZH2 inhibitors and standard chemotherapy could be a superior potential treatment for pancreatic cancer.

Conclusions: Our results demonstrate nuclear accumulation of EZH2 as a hallmark of poorly differentiated pancreatic adenocarcinoma, identify the tumor suppressor $p27^{Kip1}$ as a new target gene of EZH2, show that EZH2 nuclear overexpression contributes to pancreatic cancer cell proliferation, and suggest EZH2 as a potential therapeutic target in pancreatic cancer.

Rationale: The modality being developed is the identification of EZH2 as a potential therapeutic target. Small molecule inhibitors have been developed toward other histone methyltransferases. A small molecule inhibitor toward EZH2, could be useful for not only affecting proliferation of pancreatic cancer cells, but also making them more sensitive to widely used chemotherapeutic agents, such as gemcitabine and doxorubicin.

Keywords: EZH2, histone methyltransferase, pancreatic cancer

435 Studies Directed Toward the Prevention and Treatment of Brain Metastasis From Breast Cancer

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The incidence of brain metastases in breast cancer patients is increasing as a sanctuary site as chemo- and molecular therapies improve systemic survival. Few therapeutic strategies exist for the treatment of metastatic tumor cells in the brain where the blood-brain barrier severely limits drug access. Our laboratory has developed a quantitative model system for the study of breast cancer brain metastasis using an EGFP labeled "brain-seeking" derivative of the human MDA-MB-231 breast carcinoma cell line (231BR). We are using this model to study the biology of breast cancer brain metastasis and to preclinically test molecularly targeted agents for the prevention and treatment of breast cancer brain metastasis. Her-2 overexpression in 231-BR cells resulted in a 2.5-3 fold increase in large metastases. We tested the efficacy of the dual EGFR/Her-2 tyrosine kinase inhibitor, lapatinib, and the HDAC inhibitor, vorinostat (SAHA), in the model system. Treatment with 30 mg/kg lapatinib resulted in a 53% decrease in large metastases ($p=0.0001$), while treatment with 100 mg/kg resulted in a 50% decrease in large metastases ($p=0.0002$) in Her-2 overexpressing 231-BR cells. Lapatinib (100 mg/kg) also caused a 54% decrease in large metastasis ($p=0.0003$) in 231-BR-vector control cells which endogeneously overexpress EGFR. These results validate lapatinib as the first Her-2 directed drug to have activity against brain metastases of breast cancer in a preclinical model. Vorinostat (SAHA), a histone deacetylase inhibitor, was also tested in vivo in a prevention and a treatment model of breast cancer brain metastasis. In the prevention model, treatment was initiated 3 days after intracardaic injection of 231-BR cells. Compared with the untreated control group, vorinostat at 150 mg/kg inhibited the development of large brain metastases by greater than 60% ($p<0.0001$) and micrometastases by 30% ($p=0.017$). In the treatment model, vorinostat dosing was initiated 7 and 14 days, respectively, after injection of 231-BR cells. Compared with the untreated control group, there was a significant reduction in large metastases ($p=0.008$) when treatment was initiated on day 7. However, there was no significant effect of vorinostat when treatment was initiated 14 days after injection of 231-BR cells. These data suggest vorinostat as a new drug for the prevention of brain metastases of breast cancer and indicate a therapeutic window for treatment.

Keywords: metastasis, breast cancer, brain

436 Rational Targeting of Notch Signaling in Breast Cancer

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Background: There is strong evidence supporting a key pathogenic role of the Notch pathway in breast cancer. Expression of various Notch-related biomarkers correlates with poor prognosis in breast cancer patients. Constitutively active Notch-1, -3 and -4 cause mammary tumors in transgenic mice, and Notch-1 and -4 transform human mammary epithelial cells. Additionally, Notch is one of the pathways that control the self-renewal of "breast cancer stem cells". Several Notch inhibitors are being developed. Among these, at least two γ-secretase inhibitors (GSIs), are in early clinical trials. However, Notch signaling is notoriously context- and dose-dependent, and is regulated by a complex network of cross-talk interactions with other pathways, some of which include other potential therapeutic targets. Rational development of Notch inhibitors for breast cancer therapy will require a detailed understanding of this cross-talk in specific breast cancer subsets. This will allow: 1) the identification of disease specific pharmacodynamic biomarkers, 2) the identification of patient subsets that are particularly likely to benefit from these agents and 3) the design of rationally targeted combinations.

We have determined that: 1) In ERα+ breast cancer cells, estrogen suppresses Notch signaling by regulating the cellular distribution of Notch-1. Estrogen withdrawal, mimicking the effects of aromatase inhibitors, or SERMS, cause re-activation of Notch signaling and increase dependence on Notch for proliferation, survival and invasion. Combinations including a GSI and an anti-estrogen are synergistically effective *in vitro* and *in vivo*. We have initiated a pilot clinical trial to determine the safety and tolerability of a combination regimen including endocrine therapy and an investigational GSI, and to identify disease-specific activity biomarkers; 2) Notch-4 plays a key role in at least one model of tamoxifen resistance, and GSIs are highly effective in this model, reversing tamoxifen resistance; 3) ERα-, PR-, ErbB-2^{low} MDA-MB231 cells are highly sensitive to Notch inhibition *in vitro* and *in vivo*; 4) ErbB-2 overexpression inhibits Notch signaling by modulating membrane availability of Notch ligands. Treatment of ErbB-2^{high} breast cancer cells with trastuzumab or a tyrosine kinase inhibitor (TKI) causes re-activation of Notch signaling. Combination regimens including a ErbB-2 targeting agent and a GSI are at least additive *in vitro* and prevent tumor recurrence *in vivo.* Moreover, a GSI can reverse trastuzumab resistance *in vitro*; 5) Mammosphere formation by "breast cancer stem cells" is dramatically inhibited by GSIs.

Our data support the therapeutic investigation of Notch inhibitors in combination with: 1) endocrine therapy in ERα-positive breast cancers and in some tamoxifen-resistant breast cancers; 2) ErbB-2-targeted agents in ErbB-2 positive and trastuzumab-resistant breast cancers; Additional combinations for rational targeting of triple-negative breast cancer cells and "cancer stem cells" are being tested.

Keywords: notch, breast cancer, gamma secretase inhibitors

437 Targeting the HER Network by Combination drug Therapy to Overcome Resistance in HER2-positive Breast Cancer

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The HER network is a robust redundant network providing important proliferation and survival signals to a subset of human breast cancer. Gene amplification of HER2, a critical player in the input layer of the system, in 25% of breast cancer led to development of drugs targeting the pathway. In breast cancer, drugs that are currently in use target the receptor input layer to block the pathway. Trastuzumab binds to the external domain of HER2 and, thereby, inhibits signaling through the network. In metastatic breast cancer, the drug used alone induces responses in 30% of patients while the majority demonstrate d*e novo* resistance. In the adjuvant setting about 50% of patients with HER2 expressing tumors benefit from trastuzumab with a sizable improvement in time to progression and survival. Still many patients demonstrate either *de novo* or acquired resistance to the drug. Considering the complexity of the network, resistance to trastuzumab could develop at several levels.

One potential mechanism is an incomplete blockade of signals generated by the various dimer pairs formed by this family of receptors (HER1-4). However, other drugs that target the pathway in slightly different ways have been developed, including the tyrosine kinase inhibitors lapatinib, gefitinib, or erlotinib, or the monoclonal antibody and dimerization inhibitor pertuzumab. In preclinical models these drugs used in various combinations to more completely block signals from all heterodimers pairs is a more effective strategy than any of the agents used alone and is capable of completely eradicating some xenograft models of human breast cancer. The most effective combinations are lapatinib plus trastuzumab, or gefitinib, pertuzumab, and trastuzumab. Other xenograft and cell culture models are resistant to combination therapy suggesting alternative mechanisms of resistance.

An additional possible resistant mechanism that we focus on, stems from the crosstalk between the HER and the estrogen receptor (ER) pathways. This bidirectional crosstalk occurs at multiple levels and transmits both stimulatory and inhibitory influences. Importantly, the HER pathway via various mechanisms can downregulate the expression and/or the activity of the ER. Accordingly, inhibition of the HER pathway can upregulate the expression and/or activity of ER to provide an alternate survival pathway for the cell. Signaling from this re-expressed or re-activated ER, if left unchecked, can cause resistance to HER-targeted therapy. This molecular scenario suggests the possibility that in some breast cancers a simultaneous blockade of both HER and ER signaling pathways may be required to bypass resistance mechanisms, resulting in optimal treatment benefit.

We are now launching a phase II neoadjuvant clinical trial looking at the trastuzumab plus lapatinib combination along with endocrine therapy where applicable, together with sequential biopsies to assess the response rate (including complete pathological response) and to obtain clues for the mechanisms of action and resistance to the regimen. A future trial in the metastatic setting combining trastuzumab, pertuzumab, and erlotinib is in the planning stage.

Keywords: HER2, trastuzumab resistance, breast cancer

438 Bispecific Antibody Pretargeted Radioimmunodetection and Therapy of Pancreatic Cancer

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The dismal prognosis for pancreatic cancer requires a concerted effort to find new ways for early detection and treatment. Our group first described a monoclonal antibody, PAM4 (Gold et al., Int J Cancer. 1994 Apr 15;57(2):204-10) that binds to a unique epitope expressed specifically in pancreatic cancer mucin. ELISA testing of serum indicated the antigen was detectable in pancreatic cancer patients with a greater specificity and sensitivity than CA19-9 (Gold et al., J Clin Oncol. 2006 Jan 10;24(2):252-8), and recent immunohistology studies have detected the antigen in very early pre-cancerous lesions, but absent in normal or non-malignant diseased pancreas. Initial studies showed the feasibility of PAM4-radioconjugate targeting of pancreatic cancer xenografts in animal models, as well as their application for therapy. ^{90}Y-labeled PAM4 IgG could also be combined with gemcitabine to enhance treatment response. These studies lead to the development of a humanized PAM4 IgG that is currently under Phase II clinical investigation as a radioconjugate being used in combination with gemcitabine.

These promising data have led to the development of a second generation targeting system, namely a bispecific antibody pretargeting method. Bispecific antibody pretargeting protocols are being explored because they are able to target radionuclides to tumors with a strong signal, often rivaling the uptake of a directly radiolabeled IgG, but with much higher tumor/nontumor ratios, and do so in a rapid manner. This often allows for better therapeutic responses with less hematologic toxicity. Our system uses a novel tri-Fab, recombinant protein (TF10) that binds divalently to the tumor antigen and monovalently to a synthetic hapten, histamine-succinyl-glycine (HSG). Collaborating with 2 biotechnology firms, we have engaged in the initial preclinical testing both for detection by external scintigraphy and therapy. Imaging studies in the CaPan1 human pancreatic cancer xenograft model using an ^{111}In-labeled HSG-peptide pretargeted with TF10 have shown exceptional tumor uptake (~20-25% injected dose per gram) and tumor/nontumor ratios within 3 h of the HSG-peptide's injection (tumor to blood, liver, kidneys equaling ~1000:1, 100:1, and 10:1, respectively) (Gold et al., Cancer Res. 2008 Jun 15;68(12):4819-26). Using a similar pretargeting system against CEA (carcinoembryonic antigen) with an ^{124}I-labeled HSG-peptide, micrometastatic tumor nodules in the lungs as small as 0.3 mm in diameter were visualized (Sharkey et al., Radiology. 2008 Feb;246(2):497-507). This has provided a basis to explore additional hapten-peptides suitable for use with other positron-emitting radionuclides, such as ^{68}Ga and ^{18}F. For therapy, a ^{90}Y-labeled HSG-peptide has been tested in the CaPan1 xenograft model and found to provide excellent anti-tumor responses alone, with enhanced responses when combined with gemcitabine.

The specificity of PAM4 for pancreatic cancer and the exceptional sensitivity of pretargeting for detection and its therapeutic potential with less hematologic toxicity have provided the incentive to begin the process for developing an IND. Our prior preclinical and clinical experience using PAM4 IgG and the recent successful IND approval for the CEA pretargeting system that will begin clinical testing this year provide a strong foundation for this process. (Supported in part by R01-CA115755 from the National Cancer Institute.)

Keywords: pancreatic cancer, bispecific antibody, pretargeting radioimmunotherapy

439 Targeting Epigenetic Modifications in Breast Cancer

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Breast cancer is a consequence of an accumulation of genetic and epigenetic events. We hypothesized that therapies directed against epigenetic changes will be a useful strategy against breast cancer; here the effects of histone deacetylase inhibitors (HDACI) are addressed.

Our studies show that HDACI have multiple effects in human breast cancer cell lines. Clinically relevant HDACI such as vorinostat and LBH589 have been shown to reexpress epigenetically silenced genes such as ESR1, leading to reexpression of a functional estrogen receptor alpha (ER) protein and sensitization to the growth inhibitory effects of tamoxifen in ER-negative human breast cancer cell lines including the prototypical ER-negative MDA-MB-231 cell line. In concert downregulation of expression of the epidermal growth factor receptor (EGFR) protein is noted after vorinostat treatment. This is a consequence of altered EGFR mRNA stability as well as inhibition of downstream elements of the EGFR signaling pathway. In addition HDACI treatment of ER-negative human breast cancer cells leads to downregulation of DNA methyltransferase 1 (DNMT1), the primary maintenance DNMT; this reflects ubiquitination of DNMT1 protein leading to its proteasomal degradation. The multiplicity of effects of HDACI led us to undertake a comprehensive assessment of the acetylated lysine proteome in vorinostat-treated MDA-MB-231 cells using a stable isotope labeling method; multiple candidate proteins that might serve as biomarkers for biological effects have been identified and validation and functional studies are in progress.

In parallel the biological effects of vorinostat in women with newly diagnosed untreated primary human breast cancer are under evaluation in a preoperative “window” study. Twenty-five women with early stage breast cancer will take vorinostat 300 mg po bid for 3 days before definitive surgery and pre- and post-therapy breast cancer tissues and blood samples are collected for analysis. Thus far this therapy has been shown to be safe and collection of samples is feasible. Trial accrual is nearly complete. End points including Ki67, acetylated histones, methylation and expression of candidate genes, global transcriptional analysis, and acetylation of candidate proteins identified during the proteomic analysis will be evaluated.

In aggregate these studies will elucidate the effects of HDACI on human breast cancer cells in culture and in women and lay the foundation for rational combination studies. Supported by NIH CA 88843.

Keywords: breast cancer, epigenetic, histone deacetylase inhibitor

440 Therapeutic Sensitivities of Transgenic Mouse Models of Human Breast Cancer

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Genetically Engineered Mice (GEM) have proven to be of great value in validating the causality of specific oncogenes and tumor suppressor genes in many cancer types including human breast tumors. However, the phenotypes of some GEM models fail to correspond to their alleged human counterparts, thus complicating the use of the GEM for therapeutic testing. To address this issue, we expression profiled numerous GEM models of mammary cancer (Herschkowitz et al. 2007) and identified murine models that we consider to be good representatives of two human breast tumor subtypes: Basal-like tumors (C3-Tag), and Luminal B tumors (MMTV-Neu).

The clinical criteria for choosing the right chemotherapeutic and biological agent combination for each breast tumor subtype has yet to be experimentally determined. Our ultimate goal is to use these validated GEM models as a preclinical testing ground for new drugs, and new therapeutic combinations. As a first step towards this goal, we have tested on each model: 4 chemotherapeutics (doxorubicin, carboplatin, paclitaxel, and cyclophosphamide), 2 chemotherapy combinations (carboplatin/paclitaxel and doxorubicin/cyclophosphamide), and 2 biologically targeted agents (erlotinib and lapatinib).

The results show that although several of the single-agent chemotherapeutics were able to significantly reduce the growth rates of the tumors, no single-agent chemotherapeutic was able to elicit tumor regression. In contrast, the combinations of doxorubicin/cyclophosphamide in the MMTV-Neu model and of carboplatin/paclitaxel in the C3-Tag model were able to cause dramatic tumor regression, and even complete clinical responses in most animals as assessed by ultrasound imaging.

In the C3-Tag basal-like model, both erlotinib and lapatinib as single-agents showed a range of responses from tumor regression to progression. This suggests that heterogeneity within this genetically defined model still exists. In the MMTV-Neu model, erlotinib alone was able to cause significant tumor regression, while lapatinib caused rapid and complete tumor response in every animal tested. Additional chemotherapeutic and biological agent combination therapies are now being tested in these models. We are also investigating the effects of variations in sequence and dose scheduling. These results show that genomically selected GEM models can recapitulate findings seen in human tumors (like lapatinib responsiveness in HER2+ tumors and carboplatin sensitivity in basal-like tumors), and hopefully, they can be used to develop and refine new treatment regimens before testing in humans.

Keywords: breast, mouse, chemotherapy

441 Chemopreventive and Therapeutic Effects of Cruciferous Vegetable Constituent Benzyl Isothiocyanate Against Breast Cancer

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Breast cancer continues to be a leading cause of cancer-related deaths in women worldwide. The known risk factors for breast cancer include family history, Li-Fraumeni syndrome, atypical hyperplasia of the breast, late age at first full-term pregnancy, early menarche, and late menopause. Because some of these risk factors are not easily modifiable, other strategies for reduction of breast cancer risk must be considered. Even though selective estrogen-receptor (ER) modulators (*e.g.,* tamoxifen) appear promising for prevention of breast cancer, this strategy is largely ineffective against ER negative breast cancer. Therefore, identification of agents that are non-toxic but can delay onset and/or progression of breast cancer is highly desirable. Natural products have received increasing attention in recent years for the discovery of novel cancer preventive and therapeutic agents.

The present study was designed to determine efficacy of benzyl isothiocyanate (BITC), a constituent of edible cruciferous vegetables, against breast cancer using cellular and animal models. We demonstrate that BITC suppresses growth of cultured human breast cancer cells (MDA-MB-231 and MCF-7) regardless of their estrogen-responsiveness or p53 status by causing apoptotic cell death. On the other hand, a spontaneously immortalized but non-tumorigenic normal mammary epithelial cell line (MCF-10A) is significantly more resistant to growth inhibition and apoptosis induction by BITC compared with breast cancer cells. The BITC-induced apoptosis in human breast cancer cells is initiated by mitochondria-mediated generation of reactive oxygen species (ROS). Mitochondrial DNA deficient Rho-0 variant of MDA-MB-231 cells, generated by culture in the presence of 1 mM sodium pyruvate, 1 mM uridine and 2.5 μM ethidium bromide over a period of seven weeks, is nearly completely resistant to BITC-mediated ROS generation and apoptosis. Intraperitoneal and oral administration of BITC significantly inhibits growth of MDA-MB-231 cells implanted in female athymic mice in association with increased apoptosis in the tumor. In addition, dietary administration of 1 and 3 mmol BITC/kg diet inhibits incidence and burden of hyperplasia in MMTV-neu transgenic mice. The incidence of carcinoma in MMTV-neu mice is also markedly suppressed by dietary administration of 1 mmol BITC/kg diet.

In conclusion, the present study provides convincing preclinical data to warrant clinical investigation of BITC against breast cancer in humans. This study was supported by the National Cancer Institute grant CA129347.

Keywords: benzyl isothiocyanate, breast cancer, chemoprevention

442 Identification of RalB as a Therapeutic Target for Metastatic Pancreatic Cancer

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Pancreatic cancer remains one of the deadliest cancers. Although we have seen a great deal of success with targeted therapies for cancer treatment, too often efforts concentrate first on drug discovery, and secondarily search for cancers that will respond to a given drug. We set out to first define what therapeutic targets are important to specific groups of tumors and patients and second to determine drugs for those targets (e.g. trastuzumab treatment for Her2/Neu positive breast cancers).

To this end we have identified a group of genes that correlate with pancreatic cancer metastasis. Using significance analysis of microarray (SAM), a false discovery rate of 0.1% and an expected median false positive number of genes of 1, we identified 500 genes that were differentially expressed in 49 metastases with matched primary tumors from 13 patients and 16 primary tumors resected from patients without metastases. Our metastasis signature had a cross validation accuracy rate of 93%. In addition, we developed a Single Sample Predictor (SSP, Nearest Centroid Alogrithm) using the mean gene expression profile (i.e. centroid) for the metastases and non-metastases groups. We found that our SSP ($p=0.04$) was a better predictor of prognosis than any other traditional clinicopathological variable including lymph node status ($p=0.08$) in an independent set of pancreatic cancer patients without metastasis, suggesting that these genes may be promising targets for treatment and/or diagnosis of both primary and metastatic pancreatic cancer.

One exciting gene that we identified was RalB, a downstream effector of Ras, the oncogene that is mutated in 90% of pancreatic cancers. Despite the critical role of aberrant Ras function in promoting malignant pancreatic growth, to date, no effective anti-Ras therapies have been developed. Our previous studies in pancreatic cancer cell lines demonstrated that RalB plays a critical role in Ras-driven invasion and metastasis of pancreatic cancer. In addition, we found that RalB activation was higher in metastases compared to matched primary tumors. Taken together, our data suggests that RalB will be a clinically and functionally relevant target in pancreatic cancer. Therefore, we have identified a pharmacological inhibitor of RalB, the GGTase-I inhibitor, GGTI-2417 (Tigris Pharmaceuticals), which targets RalB.

We have firmly established the clinical and functional importance of the RalB in metastatic pancreatic cancer. We have made the critical first steps, of target validation and the identification of therapeutic candidates. Our proposed studies now enter into the most critical phase of molecular-targeted drug discovery, preclinical demonstration of anti-tumor activity and identification of biomarkers for monitoring drug activity and efficacy. Our studies establish a strong foundation upon which we can now work together to bring the anti-Ral therapies, as novel anti-Ras inhibitors to the clinic.

Keywords: pancreatic cancer, metastasis, RalB

443 Discovery and Validation of Molecular Targets, Biomarkers, and Nontoxic Interventions for Cancer Prevention

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The Gene Regulation Section, Laboratory of Cancer Prevention (LCP) is focused on understanding the early events in tumorigenesis with the hope of finding new molecular targets, biomarkers and non-toxic interventions that will prevent or significantly delay the beginning of cancer. Our work in vitro and with genetically engineered mice (GEM) has repeatedly shown that the transcription factor AP-1 can be targeted for cancer prevention and intervention (Young et al., 2003). Specifically, mice that express dominant negative c-Jun (TAM67) are protected from environmental and oncogene induced skin and breast cancer without any noted toxicity to the animals (Young et al., 2003, Shen et al., 2008). From these studies we designed a high-through put screen of the NCI-Synthetic and Natural products libraries to identify compounds that would target induced AP-1 activity without affecting cell proliferation (Ruocco et al., 2007). One such compound, NCI676914, although it inhibits AP-1 activation shows greater potency for inhibiting NF-κB activation with an IC50 of about 4 μM (Kang et al., submitted). NCI676914 reduced phosphorylation of IKKα/IKKβ significantly, resulting in a complete block of IκB-α phosphorylation. Concentrations of NCI676914 as low as 1.1 μM repressed NF-κB DNA binding and transcriptional activation of NF-κB dependent genes IL-6 and COX-2. Moreover NCI676914 inhibits tumor promoter induced transformation of JB6 cells and invasion of breast cancer cells. These results suggest that single digit micromolar-range treatment is sufficient to inhibit NF-κB transactivation required for tumor promotion and progression. High selectivity and low toxicity are valuable characteristics of this potential inhibitor against its target. In addition to identifying small molecule inhibitors of AP-1 or NF-κB, the LCP has established multiple collaborations to identify dietary factors that are beneficial for cancer prevention or intervention. The Legume Intervention Feeding Experiment (LIFE) is a collaboration with Pennsylvania State University and Texas A & M. This study is designed to identify indicators of efficacious response to bean-based diets in a clinical feeding study as well as in the laboratory with mouse models of colon carcinogenesis. We have previously shown that a high bean intake is inversely associated with advanced colorectal adenoma recurrence (Lanza et al., 2006). Moreover, mice fed a diet supplemented with navy beans or bean extracts were protected from chemically induced colon carcinogenesis (Bobe et al., 2008). Proteomic analysis of sera from these mice indicated one or more proinflammatory cytokines are reduced in the mice fed the bean-based diets (Mentor-Marcel et al., submitted). In order to identify molecular targets and biomarkers of efficacy, the LIFE study designed a feeding trial in which men at high risk for colorectal cancer were fed bean-based diets for 4 weeks followed by washout and 4 weeks of an isocaloric "American" diet. Serum and fecal colonocytes were collected before, during and after each diet period. Colonoscopy was performed before entering the study. We are analyzing the data collected from these studies.

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Keywords: drug discovery, dietary intervention, prevention

444 Enhanced Estrogen-Induced Proliferation and the Antagonizing Effect of Insulin-Sensitizing Agents Rosiglitazone and Metformin in Obese Rat Endometrium

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Objective: We tested the hypothesis that the proliferative estrogen effect on the endometrium is enhanced in obese versus lean animals, and insulin-sensitizing agents Rosiglitazone and Metformin could antagonize these estrogen effects.

Study design: Using Zucker fa/fa obese insulin resistant rats and lean controls, we examined endometrial cell proliferation after treatment with estradiol, estradiol plus Rosiglitazone, or estradiol plus Metformin. We also examined the expression patterns of certain estrogen-regulated pro-proliferative and anti-proliferative genes.

Results: No significant morphological/histological difference was seen between the obese rats and the lean rats. Estrogen-induced pro-proliferative genes cyclin A and c-Myc mRNA expression were significantly higher ($p<0.05$) in the endometrium of obese rats compared with that of the lean control. Expression of the anti-proliferative gene p27Kip1 was suppressed by estrogen treatment in both obese and lean rats, however, the decrease was more pronounced in obese rats compared to the lean littermates. In lean rats, estrogen more strongly induced the anti-proliferative genes RALDH2 and sFRP4. In obese rats, estrogen had little or no effect on these anti-proliferative genes. While estradiol-induced activation of PI3K/Akt signal pathway was similar in the endometrium of obese and lean animals, estradiol-induced Ras/Raf/ MAPK signaling was increased in obese animals. Endometrial cell proliferation as shown by Brdu incorporation was decreased by Rosiglitazone and Metformin treatment in obese animals. Enhanced estrogen induced pro-proliferative genes cyclin A and c-Myc mRNA expression was reversed by Rosiglitazone and Metformin treatment in obese rat endometrium. Pronounced activation of RAS/Raf/MAPK signaling was also attenuated by Rosiglitazone and Metformin treatment as shown by the lower phosphorylation level of Erk1/2 MAPK and S6 Ribosomal protein.

Conclusion: In this model, enhancement of estrogen-induced endometrial pro-proliferative gene expression and suppression of anti-proliferative gene expression was seen in the endometrium of obese versus lean animals. Increased activation of the ERK1/2 MAPK signaling pathway was seen in the estradiol-treated obese versus the lean endometrium. Insulin sensitizing agents Rosiglitzone and Metformin treatment reversed these hyper-proliferative effects of estrogen in obese rat endometrium.

Keywords: endometrial cancer, obesity, insulin-resistant

445 Development and Application of Biosample Reference Sets for Preliminary Clinical Characterization of Biomarker for the Early Detection of Colorectal Adenocarcinoma

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The Early Detection Research Network (EDRN) is a translational, collaborative research network devoted to discovery and early phase clinical validation of biomarkers for cancer early detection and risk assessment. EDRN Clinical Epidemiology and Validation Centers (CEVCs) serve as the clinical validation arm of biomarker discovery laboratories of the EDRN. The Great Lakes-New England CEVC has developed and implemented standard operating procedures for human biosample collection, data elements, GI organ specific data elements, and protocol specific data elements for sample annotation, and a web fronted relational database for clinical informatics capable of real time biosample tracking from multiple clinical sites, through sample processing and storage, to, ultimately, biomarker assay laboratories. For example, a reference set consisting of multiple biosample aliquots (serum, plasma, urine, buffy coat DNA) was collected from 50 human participants in each of the following groups: 1) early and late stage adenocarcinoma of the colon, 2) adenomatous polyps ≥6 mm, 3) inflammatory bowel disease, and 4) normal colonoscopy. The samples are stored at -80°C and thawed once at time of assay. These samples have been sent to six EDRN associated laboratories for preliminary characterization of early cancer detection performance. Of the 12 serum or plasma colon cancer detection biomarkers assayed to date, the sensitivities range from 10% to 78% and the specificities range from 48% to 98%.

Interpretation of biomarker performance and selection of "winners" to study in a large cross sectional validation is premised upon its proposed future clinical application, where the application determines the prevalence of cases and costs of false negative and false positive decisions. For entry into a large cross sectional validation trial of a biomarker to be used as a screen for detection of colon adenocarcinoma in combination with fecal occult blood testing, we propose a performance bar for of 65% sensitivity and 95% specificity in a standardized, SOPed reference set. None of the biomarkers assayed so far meet the criteria f for the proposed application, incremental to fecal occult blood testing. The use of a high quality biosample reference set permits rapid assessment of potential biomarkers for early cancer detection at reasonable cost.

Keywords: colorectal neoplasms, biorepository, clinical epidemiology

446 Detection of Kidney and Bladder Cancer Gene Methylation in Urine DNA

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Detection of aberrant promoter methylation of the promoter region of cancer genes by quantitative real time methylation specific PCR (qMSP) is a promising new approach for the diagnosis of cancer. The technology has been applied to the diagnosis of many types of solid tumor. Methylated alleles indicative of cancer cells can be detected in voided urine specimens from individuals with kidney or bladder cancer. DNA is isolated from the urine sediment and modified by treatment with sodium bisulfite. PCR is performed with oligonucleotide primers and a fluorescence-labeled probe specific for methylated sequence in the input specimen DNA, together with appropriate positive and negative controls. A PCR reaction readout of the number of methylated alleles is given in real time. The detection of methylated genes may have utility in determining whether patients presenting with haematuria have bladder cancer, renal cell cancer (RCC) or non-neoplastic disease and in screening urine for recurrence.

A number of feasibility studies have reported relatively sensitive and specific detection of bladder or kidney cancer gene methylation in urine DNA. The promise of gene methylation as a target for the detection of cancer has been clearly demonstrated. What is most important now is the optimization and standardization of the approach and validation of clinical utility. The criteria that determine whether a panel of methylated genes is of a high enough standard to be informative in the clinic are not as well developed for those that guide the use of new therapies for cancer treatment, however certain issues are apparent. These include: the likely need for larger panels of methylated genes in detection; optimization and standardization of specimen processing and technology for analysis; further studies of gene methylation in normal or non-neoplastic cells; improved knowledge regarding the timing of methylation of a gene during neoplastic development; and the ability for differential diagnosis of the anatomical site of origin of a tumor in a body fluid. Lastly, gene methylation-based detection of cancer must be tested in regard to sensitivity, specificity, positive and negative predictive values in a large number of well-chosen cases and controls. Our current research is designed to address these issues.

To move forward beyond feasibility studies, the EDRN Bladder Cancer Working Group (Paul Cairns PhD, Bogdan A. Czerniak MD PhD, Adi Gazdar MD, Martin G. Sanda MD, David Sidransky MD and Oncomethylome Sciences) has initiated a pre-validation study of methylation-based detection of bladder cancer. Our study will test the sensitivity and specificity in cystoscopy-proven cases and controls collected from several institutions with blinding at a contracted laboratory followed by standardized processing and analysis of methylation status at another center. Precision and reproducibility will be formally tested. The study will be completed within 12 months and the outcome will inform the progress of our detection of kidney cancer studies, future studies of methylation of all types of cancer in tissue, blood, urine or other body fluids, as well as other biomarkers in urine.

Keywords: methylation, genitourinary cancer, urine

447 Detection of Promoter Hypermethylation in Saliva as a Biomarker for Head and Neck Squamous Cell Carcinoma Surveillance

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Purpose: Hypermethylation of tumor suppressor gene promoters has been found in head and neck squamous carcinoma (HNSCC) and other solid tumors. We evaluated these alterations in saliva pre-treatment from HNSCC patients in a quantitative fashion using real-time quantitative MSP (Q-MSP).

Experimental Design: Pre-treatment saliva DNA samples from HNSCC patients were evaluated for patterns of hypermethylation using Q-MSP. Evaluated tumor suppressor gene promoter regions were selected based on a previous study regarding building of a screening panel for HNSCC in a high risk population subjects. The selected genes were: DAPK, DCC, MINT-31, TIMP-3, p16, MGMT, CCNA1.

Results: We analyzed the panel in a cohort of 72 HNSCC patients. Thirty-five of the analyzed patients (48.6%) presented with methylation of at least one of the selected genes in the saliva DNA pre-treatment. Local recurrence free survival were significantly lower in patients presenting methylated pre-treatment saliva ($p=0.005$). Pre-treatment methylated saliva was not significantly associated with tumor site ($p=0.150$) nor clinical stage ($p=0.257$). At multivariate analysis for local disease free survival detection of hypermethylation on saliva pre-treatment remained as an independent factor (HR 6.8; 95%CI 1.5-32.2).

Conclusion: We were able to confirm an elevated rate of promoter hypermethylation detected in HNSCC patients saliva using a panel of gene promoters previously described as methylated in HNSCC but not in control subjects. Detection of hypermethylation in pre-treatment saliva DNA seems to be predictive of local recurrence. This finding has potential to influence treatment and surveillance of HNSCC patients.

Keywords: head and neck cancer, methylation, surveillance

448 Human Papillomavirus (HPV) and Cervical Cancer as a Model for Translational Research: Successes and Lessons Learned

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The introduction of Pap smears/cervical cytology in western countries has greatly reduced the incidence of cervical cancer, with the U.S. cervical cancer incidence and mortality rates falling by more than 75% to 10,000 cases and 4,000 related deaths annually. In parallel, laboratory scientists, clinicians, and epidemiologists working in collaboration have established that cervical infections by ~15 cancer-associated ("carcinogenic") human papillomavirus (HPV) genotypes cause virtually all cervical cancer and its immediate precursor lesions worldwide. Based on the central role of HPV in the development of cervical cancer, two HPV technologies have been developed: HPV virus-like particle (VLP) vaccines for primary prevention of HPV infections and HPV DNA testing for detection of cervical precancerous lesions. Both technologies are robust, showing high efficacy and long-term impact, and will complement, and may ultimately replace, cytology screening as the main cervical cancer prevention methods.[1]

The Translational Research Working Group (TRWG) has established developmental pathways for "early" translational research. The translation of the etiologic role of carcinogenic HPV into two technologies provides a model for translational research. For example, the development of screening tests for the detection of HPV closely parallels the developmental pathway for **Biospecimen-Based Risk Assessment Devices**. While early laboratory research discovered HPV in cervical cancer tissue, epidemiological studies were needed to define the necessary role of HPV infection in cervical cancer and its immediate precursor lesions and the natural history of HPV vis-à-vis the timing of exposures and outcomes. Critical to these steps were the development of increasingly accurate detection of HPV to clarify these relationships. From molecular epidemiologic studies emerged several important points of understanding for developing a clinically-useful molecular screening test for HPV (for example): 1) only a fraction (<1%) of all HPV infections cause clinically-important cervical abnormalities; 2) 15-20 HPV genotypes are carcinogenic HPV genotypes but the inclusion/exclusion of certain HPV genotypes require careful consideration of impact on the clinical performance and how the test will be used; 3) an appropriate positive cutpoint must be established for optimal clinical performance i.e. maximum analytic sensitivity ≠ best clinical performance; 4) clinical performance is age dependent; 5) tests must be reliable.

Although the focus of the TRWG is early translation, "late" translational steps (e.g., target populations, demonstrations of population efficacy, and implementation strategies) must be considered in order to achieve significant cancer prevention and control. For example, cervical cancer is primarily a cancer of poor and the underserved; more than 80% of all cervical cancer occurs in developing countries. Currently-available HPV tests and vaccines are not affordable to resource-poor populations. Fortunately, in collaboration with the NCI, lower cost versions of these technologies are currently undergoing development and validation. Future efforts will need to bridge discovery with delivery to reduce the global burden of cervical cancer.

Reference: 1. Schiffman, M., Castle, P. E., Jeronimo, J., Rodriguez, A. C. & Wacholder, S. Human papillomavirus and cervical cancer. Lancet. 370, 890-907 (2007).

Keywords: cervical cancer, Human Papillomavirus (HPV), Pap smear

449 Pancreatic Cancer-Induced Diabetes Mellitus (DM): Opportunity for Risk Stratification and Early Detection

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The 5-year survival of pancreatic cancer is <5%. Surgical resection offers the only hope of long-term survival. However, since cancer-specific symptoms occur only when pancreatic cancer is at an advanced stage, >85% of pancreatic cancers are surgically unresectable at diagnosis. We have conducted a series of studies investigating the role of new-onset DM as a harbinger of pancreatic cancer. We have shown that ~85% of patients with pancreatic cancer have an abnormal fasting glucose and nearly half have DM which is frequently new-onset, i.e. of <24 months duration. In a population-based study we showed that compared to the general population, subjects with new-onset DM have an 8-fold higher risk of being diagnosed with pancreatic cancer within 3 years of meeting criteria for DM. In a recent large case-control study we found that the onset of DM precedes diagnosis of pancreatic cancer by 18-24 months. We have observed that in 57% of patients with pancreatic cancer who have new-onset DM, the DM resolves after resection of cancer, whereas DM prevalence was unchanged among pancreatic cancer patients with longstanding type 2 DM. In retrospective studies we noted that patients with pancreatic cancer appeared resectable >6 months prior to clinical diagnosis. Marked insulin resistance has been reported in pancreatic cancer-induced DM. However, ß-cell failure to compensate for lack of insulin sensitivity is essential to develop DM in insulin-resistant states. This is supported by our observation of marked decline in ß-cell function in pancreatic cancer subjects with pre-diabetes. In i*n vitro* studies using an insulinoma cell line (INS-1) we have observed that pancreatic cancer cell lines inhibit glucose-mediated insulin release.

Our data suggest that if pancreatic cancer-induced DM can be distinguished from type 2 DM, older subjects with new-onset hyperglycemia and diabetes would benefit from further screening for asymptomatic, early stage pancreatic cancer. We are developing and evaluating biomarkers which will facilitate identification of pancreatic cancer-induced DM.

Keywords: pancreatic cancer, risk stratification, diabetes mellitus

450 Pancreatic Cancer Protein Biomarkers for Early Detection

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Pancreatic cancer is a uniformly lethal disease because most patients have no symptoms until the cancer has spread and become inoperable. Biomarkers for early detection are urgently needed to improve the diagnosis and prognosis of this deadly disease. In this study, we develop methods for the comprehensive development of candidate pancreatic cancer biomarkers and the algorithm that are used to determine which biomarkers are best suited for development into a clinically useable test.

Large-scale quantitative global protein profiling experiments using different quantitative proteomics techniques and mass spectrometric platforms were performed, including ICAT and iTRAQ analyses of pancreatic carcinoma in situ (known as PanIN3), pancreatic cancer, and pancreatitis compared with normal control. These studies have led to identification of over 250 up-regulated proteins in pancreatic cancer and pre-cancer. Triage and prioritization of these biomarker candidates were then performed to prioritize candidates for further ELISA testing. Of the six ELISA tests developed so far, the 2 best performers were MMP-7 (sensitivity =78% and specificity =71%) and THBS2 (sensitivity =53% and specificity =93%) when pancreatic cancer cases were compared to unaffected clinic-based controls. In a disease like pancreatic cancer, where the prevalence of the disease is low, the specificity of the test is paramount. Thus, while THBS2 provides a modest sensitivity, the specificity is very good and over-all it outperforms the current biomarker used in pancreatic cancer called CA19.9.

In summary, proteomic analysis is providing insight into the proteins that underlie pancreatic cancer and a list of candidates that are worthy of consideration for biomarker development. Careful triage assessment of these proteins has led to a short list of candidate proteins for ELISA development. Of the six ELISA tests developed so far, two candidates have out-performed the current biomarker CA19-9 for pancreatic cancer. We plan to expand on these preliminary data and develop further ELISA tests for other biomarker candidates to achieve better sensitivity and specificity.

Keywords: pancreatic cancer, biomarker, proteomics

451 A Progress Report for a Biomarker Reference Laboratory (BRL) for Early Detection Research Network (EDRN)

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The UCLA BRL for EDRN serves as one of the laboratory to validate biomarkers. This validation process could involve technology development, quality control, refinement of assay, and high-throughput. We were involved in several validation studies, and would discuss two of these projects. A) Des-gamma Carboxyprothrombin (DCP), Alpha-fetoprotein (AFP), and Lectin-bound Alpha-fetoprotein (AFP-L3) in early hepatic carcinoma (HCC). B) Validation of salivary m-RNA as bio-markers for oral cancer.

A) The study enrolled a total of 846 patients in this study, of which 424 (50%) were cirrhotic controls without cancer and 422 (50%) were HCC cases. Of the cases, 208 (49%) were early stage HCC. DCP, AFP, and AFP-L3 levels were determined in a blinded manner using a sandwich ELISA (Eisai Co, Japan) for DCP and AFP and AFP-L3 were measured using a Liquid-phase Binding Assay (LBA) by Wako, USA. The serum levels of total AFP, DCP and AFP-L3 were significantly elevated in cases (both early and late stages) when compared to control. However, when cirrhotic controls and only early stage HCC were considered, AFP had the best AUC (0.80,95%CI:0.76-0.84) followed by DCP (0.72,95%CI:0.67-0.76) and AFP-L3 (0.66,95%CI:0.62-0.70). When cirrhotic controls and late stage HCC were evaluated, DCP had the highest AUC (0.89, 95%CI:0.86-0.92) compared to total AFP (0.84, 95%CI:0.81-0.88), indicating that DCP was more predictive of late stage HCC than of early stage HCC.

B) A panel of 7 salivary mRNAs were quantified in anonymized aliquots of saliva samples (32 oral cancer and 32 matched controls) in BRL , and Wong's lab at UCLA. RNA was isolated semi-automatically (KINGFISHER, Thermo Scientific) and transcriptomic biomarkers were quantified by a multiplex RT-PCR preamplification and subsequent singleplex real-time PCR, and all reactions were set up using the BioMek 3000 liquid handling platform into 96-well plates. Data (Ct numbers and relative copy numbers) were obtained and analyzed by statisticians at EDRN Data Management and Coordinating Center at the Fred Hutchinson Cancer Center. Three genes for EDRN-BRL and all 7 from the UCLA lab, were significantly elevated in oral cancer samples ($p<0.05$). The predictive power and standardized measurement of these salivary oral cancer biomarkers were highly consistent and reproducible. Importantly, they were independently validated by EDRN BRL laboratory.

In summary, A) combination of AFP and DCP improves the sensitivity for early stage HCC to 71% from cirrhotic controls. B) We also demonstrated that the predictive power and standardized measurement of these salivary oral cancer biomarkers were highly consistent and reproducible.

Keywords: EDRN, validation studies, biomarker reference lab

452 Development of an RNA Sensor Platform for Detection of Circulating Tumor Cells

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We are developing a chip-based RNA sensor, with an initial application for detection of circulating tumor cells. Circulating tumor cells are harvested from blood using a simple porous membrane gradient centrifugation device, and RNA is extracted. Target RNAs from specific cancers are selected from the literature, and library selection is performed to optimize antisense oligonucleotide (ASO) binding sites. Detection is based on a "hybridization sandwich", with one ASO_1 covalently attached to silicon or rhodium nanowires, and the other ASO_2 covalently attached to a 40nm Au particle. Formation of the ASO_1-RNA-ASO_2 sandwich induces a shift in the resonance frequency of a nanowire cantilever, which is measured optically. We have optimized ASO sites for a number of target RNAs for prostate, breast, and melanoma cancers, developed conditions that allow single nucleotide mismatch discrimination, shown that the ASO-derivatized nanowires remain functional following assembly and on-chip integration, developed the ability to assemble derivatized nanowires at predetermined chip locations with high yield, and shown that femtogam-level formation of each target RNA sandwich complexes induce a shift in resonance frequency that can be easily detected. The platform is currently being extended to allow quantification of proteins, by replacing the ASOs with aptamers, and the next phase of development will include transitioning to direct electrical readout of binding events. We have initiated clinical trials with melanoma patients; samples will be analyzed using conventional techniques (QPCR, ELISA) for benchmarking, and the balance of the samples will be analyzed using the RNA sensor platform when it is available.

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Keywords: sensor, nanotechnology, CTCs

453 Validation of Ovarian Cancer Biomarkers in Prediagnostic Specimens From the PLCO Trial

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In 2005, Daniel Cramer representing Early Detection Research Network (EDRN) and Nicole Urban representing Ovarian SPORE sites submitted an application to leadership of the Prostate, Lung, Colon, and Ovarian (PLCO) Cancer Screening Trial with the goal of identifying a panel of biomarkers that could be applied to PLCO specimens to detect preclinical disease. The project consisted of two phases. In phase I, the participating sites assembled a set of sera from women with ovarian cancer and benign diseases as well as from healthy general population controls not undergoing surgery. Sera from cases and benign disease controls were collected pre-operatively. Using this standard set of specimens, multiple markers were tested by laboratories at four different sites: Fred Hutchinson Cancer Research Center under the direction of Nicole Urban, Harvard's Massachusetts General Hospital Tumor Marker Lab under the direction of Pat Sluss, MD Anderson Hospital under the direction of Robert Bast, and the University of Pittsburgh under the direction of Anna Lokshin. Data were submitted to the EDRN's Data Management and Coordinating Committee (DMCC) for estimation of marker and assay performance followed by unblinding of a training set and then the full set for release to study sites. The plan was that, after selection of the final markers, the PLCO would release specimens from the PLCO trial to test the ability of the markers to detect preclinical disease in the second phase of the study.

The first phase is now complete. Nearly 1000 case and control specimens (160 ovarian cancers [half early stage], 160 benign disease, 480 general population, 84 identical replicates, and 38 pairs of serial specimens) were assembled and sent to Dr. Godwin's laboratory at Fox Chase Cancer Center for re-aliquoting, blinding, and shipping to the four assays sites. The four sites completed assays on over 50 different markers and submitted their data to the DMCC. Using half of the specimens as a training set, various marker panels were selected for evaluation in the test set; final performance of all markers was evaluated in the entire set. At 98% specificity (based on general population controls), the top ten performing markers (based on sensitivity for all cases) were: CA 125 (at 66% sensitivity), HE4 (50%), CA15.3 (39%), CA72.4 (34%), Transthyretin (34%), B7H4 (30%), IGFBP2 (28%), Mesothelin (28%), Human Kallikrein 6 (28%) and Cytokeratin 19 (24%). Markers from all four sites were represented on this list.

PLCO leadership has now released sets of PLCO trial specimens to the four assay sites. The above markers and others will be measured in sera from a set that includes 119 cases with blood samples that were collected, on average, 6 months prior to diagnosis, 952 controls matched by age and timing of specimen collection, and 90 quality control specimens. Assays have been completed by all laboratories and results returned to the PLCO, who will unblind the data for about half of the specimens in a test set to allow laboratories to further refine their screening algorithm in an iterative process for retesting in a validation set. Whether or not additional markers can improve upon the sensitivity and specificity of CA125 for the early detection of ovarian cancer should be known in late 2008 or early 2009.

Keywords: ovarian cancer, early detection, serum markers

454 Advances in Understanding Precursor Lesions for High-Grade Serous Cancers

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Background: An understanding of the histologic and molecular features of lesions which precede cervical, colon, and certain other cancers has advanced understanding of the pathogenesis of these cancers but has been lacking for invasive serous cancers of the ovary. Research has focused on the ovarian surface epithelium or surface-derived inclusion cysts as the likely sites of origin, but recently attention has shifted to the Fallopian tubes. Following our initial description of the fimbriated end of the Fallopian tubes, rather than the ovarian surface, as the dominant site of origin for occult cancers in BRCA carriers, we have extended these studies to show the model may pertain to high grade serous tumors of apparent "sporadic"origin. In our current series of investigations, we have sought to identify the sequence of epithelial changes that may occur in the Fallopian tubes in its possible progression to high grade serous cancers of the ovary.

Methods: We established a protocol for careful serial sectioning and examination of the Fallopian Tube fimbria (SEE-FIM) and have been applying this to women undergoing risk reduction salpingo-oophorectomy, women with serous carcinoma without a known mutation, and selected women undergoing hysterectomy and salpingo-oophorectomy for benign disease. The histolologic studies have concentrated on identifying a range of epithelial alterations that have in common strong nuclear immunostaining for p53 in contiguous epithelial cells as well as a high proliferative index as measured by staining for MiB1 (corresponding to Ki67). We have characterized this precursor spectrum and, in a preliminary study, determined the risk factors for these early changes.

Results: The earliest lesion in the Fallopian tube appears to involve benign-appearing secretory (rather than ciliated) cells that predominates in the fimbria and consists of intense p53 staining with evidence of DNA damage and frequently, p53 mutations (p53 signatures). The p53 signature is present in equal frequency (~30%) in women irrespective of genetic risk. A progression of increasing cellular atypia and DNA proliferation by MiB1 Index could be documented which culminated in serous tubal intra-epithelial carcinoma (STIC), considered a non-invasive but potentially malignant form of serous carcinoma. STIC is more common in women with BRCA mutations. Transitions between benign and malignant categories have been documented. Moreover, in a recent epidemiologic study, we have shown that the p53 signature shares with ovarian cancer certain risk factors, including older age at first birth and decreased parity.

Conclusion: This description of a continuum of epithelial changes in the epithelium of the fimbriated portion of the Fallopian tubes establishes steps in a model pathway that begins in the secretory epithelium of the Fallopian tubes with DNA damage and p53 mutations and ends with serous tubal intraepithelial carcinoma and eventually to high-grade serous ovarian cancers. By understanding and progressively defining this sequence, we hope to expand the opportunities for screening and early intervention of pelvic serous cancer.

Keywords: ovarian neoplasm, fallopian tubes, immunohistochemistry

455 The Early Detection Research Network (EDRN) Biomarker Validation Studies—Design and Execution

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The Early Detection Research Network (EDRN), NCI funded and investigator driven, has the mission to evaluate biomarkers for their clinical utilities in cancer risk prediction, diagnosis, early detection, and prognosis. The Data Management and Coordinating Center (DMCC) for EDRN provides support on network coordination logistics, coordinating network biomarker validation studies, developing statistical methodology relevant for biomarker evaluation, statistical services, and informatics.

We proposed PRoBE study design, Prospective specimen collection and Retrospective Blinded Evaluation, for pivotal definitive evaluation of the accuracy of a classification biomarker. Four key components of PRoBE study design include *the Clinical Context*, *Performance Criteria*, *the Biomarker test*, and *Study power and termination*. Some EDRN validation studies were used as examples to elucidate PRoBE design. Alternative designs and strategies were contrasted to illustrate the merit of PRoBE design.

DMCC developed a web-based Validation Study Information Management System (VSIMS) to facilitate study management, coordination, and encourage use standardized Common Data Elements. VSIMS has key modules for such tasks as data entry, eligibility confirmation, specimen tracking, reporting, study communication, secure data transfer, and administrative management. FDA Compliant Process was implemented in one EDRN validation study.

DMCC biostatisticians developed statistical methods for biomarker evaluations including predictiveness curve to display disease risk, prognostic marker methods, covariate and matching in ROC analysis, group sequential design for biomarker validation study, spectrum data process methods, and functional data analysis method for high dimensional data.

Reference: Pepe MS et al. JNCI in press.

Keywords: PRoBE, VISIMS

456 Ovarian Adenocarcinomas in the Laying Hen and Women Share Similar Alterations in p53, ras, & HER-2/neu

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Background: In order for an ovarian cancer animal model to yield insights that are likely to have a meaningful impact on the prevention or treatment of women with the disease, it is important that the model recapitulates as many aspects of human ovarian cancer as possible. Ideally, ovarian cancers in the model should be adenocarcinomas that arise in the ovarian surface epithelium, and have an intraperitoneal spread pattern similar to human ovarian cancer. Preferably, the neoplastic process should arise in the mature ovary, and not be subject to abnormal influences present during embryologic development. In order to account for the impact of the host on tumor growth and response to therapy, the animal should have intact immunity. In addition, the tumors that develop should have a genetic profile similar to human ovarian cancer. Finally, for the purpose of chemoprevention research, it is ideal that the tumors have a long latent phase. The chicken ovarian cancer animal model incorporates all of the attributes described above and poses an attractive alternative to genetically engineered mouse models. Ovarian tumors occur with high frequency spontaneously, in the absence of any manipulation. Thus, the developing ovary in the chicken is not subject to the abnormal influences associated with tissue-specific promoter-driven oncogenes. In addition, the chicken model shares many characteristics of human ovarian cancer. Ovarian cancers arise in the adult, mature ovarian surface epithelium in animals that have intact immunity. Tumors develop after a long latent phase, making the model well suited for investigation of chemopreventive strategies. Similar to human ovarian cancer, tumor incidence is impacted by the number of lifetime ovulatory events, and progestins confer chemopreventive effects. Finally, as we report here, a number of genetic features parallel those in human ovarian cancer.

Objectives: In this study, we sought to examine alterations in the p53 tumor suppressor gene and the ras and HER-2/neu oncogenes in chicken ovarian cancers to determine if these tumors have genetic alterations similar to those in human ovarian adenocarcinomas. The p53 tumor suppressor gene is frequently altered in human ovarian cancers, and the frequency of alteration has been shown to correlate with the number of lifetime ovulatory events predating the cancer. In contrast, *ras* is rarely mutated except for tumors of mucinous histology. Finally, HER-2/neu is overexpressed in a proportion of human ovarian cancers, and overexpression has been shown to be associated with more aggressive tumors and worse clinical outcome.

Methods: Mutations in the p53 tumor suppressor gene and the H- and K-*ras* oncogenes were assessed by direct sequencing in 172 ovarian cancers obtained from four year-old birds enrolled at age two in two separate two-year chemoprevention trials. Birds in trial B had approximately twice as many lifetime ovulations as those in trial A. Immunohistochemical staining for the HER-2/neu oncogene was performed on a subset of avian ovarian and oviductal adenocarcinomas.

Results: Alterations in p53 were measured in 48% of chicken ovarian cancers. Incidence of p53 alterations varied according to the number of lifetime ovulations, ranging from 14% in the trial A to 96% in trial B ($p < 0.01$). No mutations were seen in H-ras, and only two of 172 tumors (1.2%) had K-ras mutations. Significant HER-2/neu staining was noted in 10/19 ovarian adenocarcinomas, but only 1/17 oviductal adenocarcinomas. Moreover, Her-2/neu staining was more commonly observed in large ovarian tumors.

Conclusions: Similar to human ovarian cancers, p53 alterations are common in chicken ovarian adenocarcinomas, and correlate with the number of lifetime ovulations. Ras mutations are rare, similar to high-grade human ovarian cancers. HER-2/neu overexpression is common, and may represent a marker to exclude an oviductal origin in cancers involving both the ovary and oviduct. These data support the chicken animal model for ovarian cancer research.

Keywords: ovarian neoplasms, Gallus domesticus, p-53

457 Demonstration of Performance Qualification of the Microsatellite Marker Assay for Detection of Bladder Cancer

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Current practice in monitoring superficial bladder cancer is based on cystoscopy and urine cytology every three months for two years and then annually by a radiographic evaluation of the upper urinary tract. The sensitivity and specificity of urinary cytology are 25-50% and 90-100%, respectively. The sensitivity and specificity of cystoscopy is 90-100% and 75%, respectively. However, cystoscopy is a very invasive procedure. Thus there is a need to improve the current practice of bladder cancer detection and surveillance. Recent data suggests that analysis of microsatellite instability in urinary sediment can detect bladder cancer as early as 18 months prior to clinical diagnosis with high sensitivity and specificity. The Early Detection Research Network (EDRN) is sponsoring a validation study of a new diagnostic test, based on microsatellite (MSA) analysis of paired cancer and control specimens, for early detection of recurrent superficial bladder cancer. A panel of 15 polymorphic microsatellite markers was chosen for detection of allelic losses using three multiplex and one singleplex reactions. Assay qualification included a series of five blinded, independent validation studies by two independent laboratories (CBI and UMB). Over the course of the first four rounds qualifications, overall concordance in loci calls ranged from 78% (round 1) to 87% (round 4). Investigation by both laboratories revealed that strict quantitative measures for peak interpretation, uniform reagents that have been stringently quality controlled, and establishment of a singleplex repeat test to confirm single positive results resulted in improvements in concordance between the laboratories. In the final blinded test of 20 paired samples (blood and urine from each patient) the concordance between the laboratories, in each locus of each specimen (either case or control) was 92%; the concordance on loci that were appropriate for evaluation by both laboratories was 96%; and the overall discrimination between cases and controls, through application of the panel rule, was 100% (20/20). The successful completion of this qualification study demonstrates: 1) necessity for highly detailed standard operating procedures (SOP) that define all aspects of the assay for use by more than one laboratory, 2) performance of pre-qualification unblinded parallel studies by both laboratories that insured consistency in interpretation and equipment performance; and 3) clear definition of interpretation guidelines.

Keywords: detection of bladder cancer, microsatellite marker assay, performance qualification

458 A Signal-Dependent Organ-Restricted (SIDOR) Approach For Discovery Of Candidate Renal Cell Carcinoma (RCC) Biomarkers

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The incidence of renal cell cancer (RCC) is increasing in the United States while novel targeted therapy present a new set of clinical questions. A reliable panel of blood RCC biomarkers of disease activity could guide therapeutic and preventive interventions, and serve as surrogate end-point in clinical trials. At this juncture, a reliable biomarker for RCC has not yet been established.

To discover candidate RCC biomarkers we devised a novel approach. First, we compared the gene expression profile of human RCC cell lines deficient in the von Hippel-Lindau tumor suppressor gene (VHL) to their isogenic pair in which VHL has been reconstituted. Of the genes upregulated in the absence of VHL we selected the ones with relatively restricted normal adult tissue expression outside of kidney as potential biomarkers for RCC. This approach relies on a novel signal dependant and organ-restricted (SIDOR) algorithm for biomarker detection and led to the identification of genes as potential biomarkers for clear cell RCC: carbonic anyhdrase 9 (CA9), carbonic anyhdrase 12 (CA12), EGLN homolog 3 (EGLN3), fatty acid binding protein 6 (FABP6), hypoxia inducible gene 2 (HIG2), peripheral myelin protein 22 (PMP22), paraneoplastic antigen 2 (PNMA2) and tumor necrosis factor (ligand) superfamily 7 (TNFSF7). Next, we showed that the message of these cell-derived biomarkers is upregulated in clear cell RCC tumors compared to matched normal renal parenchyma. To validate this approach and obtain information for the individual performance of each candidate biomarker we first focused on CA9. We used an anti-CA9 antibody (M75)-based ELISA test to measure CA9 levels in blood obtained before and after nephrectomy for clinically localized disease in patients with: 1) clear cell RCC, 2) papillary and chromophobe RCC or oncocytoma, or 3) benign kidney lesions and we compared these samples to blood drawn from normal control individuals. We observed a significant ($p < 0.006$) decrease in the blood levels of CA9, after nephrectomy for localized disease, in the majority of patients with clear cell RCC (57%). In contrast, patients with non-clear cell RCC, benign disease or having undergone debulking nephrectomy for metastatic disease had no decrease in CA9 blood levels after nephrectomy. Longitudinal follow up measurements of CA9 levels in a small group of patients indicated that rising CA9 levels correlate with disease progression. We currently evaluate the rest of the RCC biomarkers by developing antibodies and immunoassays and by signature specific proteomic analysis of their levels in the plasma of RCC patients and controls.

Keywords: renal cell carcinoma, biomarkers, early disease detection

459 Identification of Serum Biomarkers of Early-Stage Pancreatic Neoplasia Using Genetically-Defined Mouse Models of Pancreatic Cancer

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Infiltrating pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States. It is almost uniformly fatal, with an overall five-year survival rate of less than five percent. The high mortality rate associated with PDAC is primarily due to the late stage at which disease is diagnosed and the resistance of pancreatic cancer to traditional chemotherapy and radiotherapy treatment approaches. These observations highlight the critical need for early detection of pancreatic neoplasia at a stage where surgical resection can provide a curative treatment option. Toward this end, we are utilizing state-of-the-art mass spectrometry and genetically engineered mouse models representing preinvasive or early, organ-confined stages of pancreatic neoplasia to identify a comprehensive set of serum biomarkers that can be used in combination to prospectively diagnose early neoplasic disease in humans. Multidimensional MALDI-TOF MS has been used to characterize changes in the serum proteome of Pdx1-Cre control animals at 1-, 2- and 4-months of age as compared with age- and histopathology-matched mice of the genotypes Pdx1-Cre;$Kras^{G12D}$, Pdx1-Cre;$Kras^{G12D}$;p16Ink4a$^{-/-}$, and Pdx1-Cre;$Kras^{G12D}$;p53$^{-/-}$. By this approach, we have been able to identify age-associated, genotype-specific changes in the serum proteome that are currently being evaluated for their sensitivity and specificity to detect early pancreatic neoplasia in humans. The goal of these studies will be the development of an antibody-based diagnostic assay that can be used to prospectively genotype and screen patients at high-risk for pancreatic cancer progression.

Keywords: pancreatic ductal adenocarcinoma, serum biomarkers, proteomics

460 Discovery and Validation of Lung Cancer Specific Monoclonal Antibodies

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There is overwhelming evidence that early and specific detection of primary lung cancer and cancer recurrence has a critical impact on the selection of therapy and ultimately on survival. Sensitive new plasma derived protein biomarkers will be vital for the development of diagnostic methods. Immunoassays are well established as valid diagnostic approaches; however, there is at present a lack of antibody reagents to protein markers in blood. Under an R21 grant, we are developing technology to address this critical need. We have adopted a three stage plasma depletion and normalization strategy to enrich for lower level potential protein markers. The processed plasma containing the natural antigen is then used for immunization of mice to generate thousands of hybridomas with potential disease specific monoclonal antibodies. A high throughput ELISA assay has been developed to screen the mAb library to discover biomarker mAbs using plasma samples of diseased vs. matched controls. In this presentation, we demonstrate the power of this strategy in two areas: (i) to discover and validate (cohort of 300 subjects) disease specific multivariate diagnostics for stage II-IV lung cancer and (ii) we show that the approach detects specific posttranslational modifications. The assays can then be used to develop new diagnostics, which we plan to complete in 2009.

Keywords: biomarker, monoclonal antibody, lung cancer

461 Molecular Targeting of Kidney Cancer Gene Pathways

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von Hippel Lindau (VHL): VHL Gene Clear cell renal carcinoma is characterized by mutation of the VHL gene. PVHL forms a heterotirmeric complex with elongin C, elongin B and Cul-2 to target hypoxia inducible factors (HIF1/2-α) for ubiquitin mediated degradation. VHL -/- clear cell renal carcinoma over expresses the EGF receptor and TGF alpha. Both HIF1-α and the EGF receptor are potential targets for molecular targeting in clear cell kidney cancer and agents that target both are being evaluated in vitro and in vivo. 17AAG (disrupts HSP90/HIF1α) and AZ6474 (targets VEGFR and EGFR) are being evaluated in vitro and in-vivo and in a clinical trials in patients with germline VHL mutation. Clinical trials are pending evaluating other agents which target this pathway in VHL and in sporadic clear cell renal carcinoma.

Hereditary Papillary Renal Carcinoma (HPRC): c-Met Gene C-Met is the HPRC gene the gene for HPRC and for a number of sporadic papillary renal carcinomas. The HPRC c-Met mutations are activating mutations in the tyrosine kinase domain of the gene. Agents which target the intracellular and extracellular portion of the gene and the ligand are under study in preclinical as well as clinical trials.

Birt Hogg Dubé (BHD): BHD Gene We have recently identified a new form of hereditary renal carcinoma (BHDS) associated with cutaneous tumors and lung cysts and identified the gene for this disorder. We have shown that the BHD gene has the characteristics of a tumor suppressor gene in the LKB1/AMPK/mTOR pathway. Agents targeting the BHD gene pathway are being evaluated in BHD -/- knockout models and clinical trials

Hereditary Leiomyomatosis Renal Carcinoma (HLRC): Fumarate Hydratase Gene We are currently studying families with HLRC, a new hereditary form of renal carcinoma. HLRC patients are at risk for uterine and cutaneous leiomyoma and a particularly aggressive form of renal cell carcinoma. Alteration of the HLRC gene, the Krebs cycle enzyme fumarate hydratase (FH) have been found to result in increase in HIF1α and HIF2α via a VHL-independent mechanism involving inhibition of prolyl hydroxylase. A trial targeting FH gene pathway is pending.

Keywords: kidney, genes, hereditary

462 Development of a Multimarker Assay for Early Detection of Ovarian Cancer

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Clinical outcome in ovarian cancer is very likely to be improved by early detection. Eighty eight candidate serum biomarkers putatively associated with epithelial cancerogenesis were analyzed in sera from healthy women and from patients with ovarian cancers (stage I-II and III-IV), benign pelvic tumors, and breast, colorectal, and lung cancers, using multiplex xMAP^{TM} bead-based immunoassays. A training set including sera from ovarian cancer patients (34 stage IA and 43 stage IB-II) and 153 healthy women was analyzed with logistic regression and cross-validation to identify an optimal panel of biomarkers for discriminating early stage ovarian cancer cases from healthy controls. The multimarker panel that provided the highest diagnostic power, 92% sensitivity (SN) at 98% specificity (SP) was comprised of 4 biomarkers: CA 125, EGFR, HE4, and VCAM-1. For comparison, the sensitivity of CA 125 alone for early stage disease was 58% at 98% SP. This model was applied to an independent blinded validation set consisting of sera from 34 patients with stage IA and 52 patients with stage IB-II ovarian cancer, 105 patients with stage III-IV ovarian cancer, and 103 healthy women providing unbiased estimates of 90% SN for stage I-II and 91% SN for stage III-IV cases at 98% SP. This panel was selective for ovarian cancer showing SN=36% for cases with benign pelvic disease, SN=8% for breast cancer, SN=4% for colorectal cancer, and SN=45% for lung cancer. We conclude that a panel of CA 125, EGFR, HE4, and sVCAM-1, after further validation, could serve as an initial stage in a screening strategy for epithelial ovarian cancer. Additional biomarkers could be utilized to further improve cancer selectivity of this panel.

Keywords: ovarian cancer, early detection, multiplex serum biomarkers

463 Detection of Immune-Related Serologic Biomarkers Prior to Lymphoma Diagnosis

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Serum levels of B cell-stimulatory molecules (IL6, IL10, sCD23), and molecules associated with B cell activation (sCD27, sCD30, IgE, CRP) are being examined in nested case-control studies of non-Hodgkin's lymphoma (NHL) or Hodgkin's lymphoma (HL). These include studies that are nested in the Department of Defense Serum Repository (DoDSR) cohort and in the Multicenter AIDS Cohort Study (MACS). Our central working hypothesis is that elevated B cell activation, as evidenced by increased serum levels of B cell-stimulatory cytokines or molecules associated with B cell activation, precedes the appearance of these B cell cancers.

In a study of HL in the DoDSR, we identified 103 HL cases and 206 matched controls with multiple serum samples collected up to the time of diagnosis, and assessed serum levels of IL6, IL10, sCD30, and IgE. Significantly elevated ($p<0.05$) serum sCD30 and IL6 were seen up to 2 years preceding HL diagnosis. sCD30 serum levels were significantly elevated in cases at 0-3 months (case median = 134 units/ml, control median = 44 U/ml), 3-12 months (case median = 86 U/ml, control median = 45 U/ml), and 1-2 years (case median= 53 U/ml, control median= 43 U/ml) prior to HL diagnosis. Serum levels of IL6 were elevated at 0-3 months (case median = 5.6 pg/ml, control median = 0.9 pg/ml), 3-12 months (case median = 2.9 pg/ml, control median = 0.9 pg/ml), and 1-2 years (case median = 1.1 pg/ml, control median = 0.8 pg/ml) pre-HL diagnosis. No significant case-control differences were observed at >2 years pre-HL diagnosis. IL10 and IgE were not significantly different in cases and controls.

In a nested case-control study of AIDS-NHL in the MACS, we assessed pre-AIDS-NHL serum levels of several B cell stimulatory cytokines and/or molecules associated with B cell activation (IL6, IL10, sCD23, sCD27, sCD30, CRP, IgE) in longitudinal serum samples collected at up to three time points prior to lymphoma diagnosis (3-5 years, 1-3 years, and <1 year pre-AIDS-NHL) in 181 AIDS-NHL cases, and equivalent time points in 181 HIV-infected lymphoma-free controls, matched to cases on actual or putative length of infection with HIV. Conditional logistic regression analyses were adjusted for change in CD4 T cell number over the course of HIV infection prior to lymphoma diagnosis. AIDS-NHL cases showed significantly elevated serum levels of sCD30 at all three time points preceding AIDS-NHL, and increased sCD27 and IL6 at 1-3 years and <1 year pre-NHL. A significantly increased frequency of detectable IL10 was observed among the cases, but only at <1 year pre-NHL.

These results indicate that elevated serum levels of several molecules associated with B cell activation precede the diagnosis of HL and AIDS-NHL, often by several years. This suggests that chronic B cell activation precedes the development of these B cell cancers. Additionally, the increasing levels of these molecules seen as lymphoma diagnosis is approached suggest that these biomarkers may also reflect tumor burden. Ongoing work is aimed at defining nucleic acid tumor markers that are clone-specific, with the intent of better defining the time (pre-clinical diagnosis) at which these cancers are first detectable.

An ongoing nested case control study (funded by R01-CA121195), utilizing the DoDSR, aims to define longitudinally serum levels of these and other B cell activation-associated molecules preceding NHL diagnosis. In preparation for this study, we are assessing Luminex-based high-sensitivity multiplex assays for cytokines and immune activation molecules. Other ongoing studies aim to define the expression of these markers pre-AIDS-NHL in the Women's Interagency HIV Study (WIHS) cohort, and also, to define the prognostic value of these and other biomarkers post-lymphoma diagnosis, in work being carried out in the AIDS Malignancies Consortium (AMC).

The views expressed are those of the authors and should not be construed to represent the positions of the Department of the Army or Department of Defense.

Keywords: lymphoma, B cell, cytokine

464 Ovarian Cancer Biomarker Validation Studies Guide New Discovery Strategies

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Objective: We have been developing approaches to systematically identify tumor-derived proteins present in the blood of women with cancer but absent in the plasma of women without ovarian cancer. We hypothesize that such markers will provide the highest sensitivity and specificity owing to the fact that their background variation in a cancer-free population is minimal.

Background: Our objectives follow from interpreting the results of several biomarker validation and discovery studies. The validation studies have measured dozens of ovarian cancer biomarkers in longitudinal samples of blood from ovarian cancer cases collected months and years prior to diagnosis, along with matched controls. Our discovery studies have interrogated cases and controls (including pre-clinical samples) using both antibody array and mass-spectrometry proteomics. These results together characterize the human plasma proteome heterogeneity and lead us to conclude that existing ovarian cancer markers, and in general any protein endogenous to women without cancer, are incapable on their own of identifying the smallest tumors. Moreover, new discovery strategies are needed to identify biomarkers because existing proteomics approaches are biased toward identification and measurement of highly abundant endogenous proteins.

Methods: We have applied several strategies to identify ovarian cancer biomarkers that have low background among the control population. One strategy includes the use of recombinant antibody libraries in principled selection (subtraction) strategies. Another uses high throughput short-read sequencing and bioinformatics to discover novel splice junctions in cDNA in ovarian cancer (OC) cells and then uses targeted proteomics strategies to confirm the presence of their associated peptides in clinical samples.

Results: With these strategies we have been able to identify peptides or proteins that are (a) present in the cancer cells of women with epithelial ovarian cancer, and (b) absent in the epithelial cells of ovarian cancer-free women (including healthy women and women with benign ovarian tumors). These proteins are presently being evaluated in clinical materials (including ascites fluid and plasma) for a role in imaging or blood-based detection strategies.

Conclusion: The heterogeneity of the human plasma proteome presents a sizable obstacle for identifying small ovarian or other malignant tumors. By assembling complementary platforms it is feasible to design new discovery strategies that cope with this heterogeneity and identify markers with high signal that also have low background variation, or noise. This strategy shows promise for ovarian cancer diagnostics but can be applied to any cancer site.

Keywords: proteomics, genomics, early detection

465 Profiling Serum Antibodies With Carbohydrate Antigen Arrays

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Antibodies are a key component of the human immune system. Changes in the repertoire of serum antibodies can occur when individuals are immunized or exposed to a pathogen, or during the onset and progression of diseases such as cancer. Methods to detect antibodies can be useful for identifying diagnostic antibodies and for the evaluation of immune responses in the development of vaccines. As a result, many strategies have been developed to detect antibodies and monitor changes in antibody levels. Most methods have focused on evaluating antibodies that bind protein antigens; however, human serum contains a diverse collection of carbohydrate-binding antibodies as well. Our group has developed a carbohydrate antigen array for the high-throughput evaluation of carbohydrate-protein interactions. The array contains over 128 different neoglycoconjugates and glycoproteins spotted on a glass slide using a robotic microarrayer. We have previously used the array to evaluate the specificities of antibodies and lectins used routinely to monitor expression of carbohydrate tumor antigens. Currently, we are using the array to profile the repertoire of anti-carbohydrate antibodies in human serum to discover new cancer biomarkers and to evaluate antibody responses generated with carbohydrate-based cancer vaccines.

Keywords: carbohydrate microarray, serum antibodies, biomarkers

466 Mesothelioma Biomarkers: Discovery and Validation

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Mesothelioma (MM) is an asbestos related orphan disease for which therapeutic options remain problematic. Two promising biomarkers, Soluble Mesothelin Related Protein (SMRP) and Osteopontin (OPN), have been investigated by the NYU EDRN Mesothelioma Biomarker Laboratory. SMRP data originated from Australian studies and OPN as a MM biomarker was discovered through pathway based genomic discovery. Initial validation of these original observations was performed from 2005-2007 using completely different cohorts of individuals.

SMRP was evaluated in serum from MM patients (n = 90), lung cancer (LC) patients (n=174), and in age and tobacco-matched AE individuals (n=66) using the MesoMark™ ELISA kit (Fujirebio Diagnostics). Receiver operating characteristic curves (ROC) were used to define true and false positive rates at various cut-offs. Mean serum SMRP levels were higher in MM compared to LC (9.47 $\pm$ 3.39 nM [mean +/- SEM] vs 1.95$\pm$0.44 nM, p=0.029), and Stage I MM SMRP levels (n=12; 2.09$\pm$0.41 nM) were significantly higher than those in AE individuals (0.99 $\pm$ 0.09 nM, p=0.02, respectively). Stage 2-4 SMRP serum levels were significantly higher (10.61$\pm$3.89 nM, p=0.03) than those for Stage 1. *The area under the ROC (AUC) was 0.805 for differentiating MM and AE, cut-off = 1.2 nM (sensitivity = 76.7%, specificity = 72.7%).* The positive predictive value was 69% and negative predictive value was 79.8% for serum.

For OPN, we investigated whether plasma OPN was comparable to serum and whether plasma OPN was a prognostic marker for MM. Plasma OPN from 39 MM (mean age 63+ 8.4 years;9 females, 30 males; 11 Stage I/II, 28 Stage III/IV; 21 having surgical cytoreduction) and from 79 AE individuals (mean age 63+ 10.6 years; 9 females, 70 males) was measured with the Research and Diagnostics (R&D, Minneapolis MN) and Immuno-Biological laboratories(IBL,Minneapolis, MN) kits. Survival was estimated using Kaplan-Meier curves; comparisons between groups are based on log rank chi-square tests. Hazard ratios and 95% confidence intervals were estimated from Cox proportional hazards models. A formal cut point analysis was performed using the maximum chi-square with p value adjustment method to determine the OPN values that were most strongly associated with survival. *The area under the ROC curve was 0.93 (R&D, cutpoint corresponding to a sensitivity of 0.91 and false positive rate of 0.23 = 59.6 ng/ml) and 0.96 (IBL, cutpoint corresponding to a sensitivity of 0.91 and false positive rate of 0.10 =132.6 ng/ml).* The median overall survival for all 39 MM was 11 months (95% CI: 5, 13) with a 37 month median overall follow up for survivors. Patients with OPN levels greater than or equal to 212.6 ng/ml had 5.7 (95% CI: 2.4, 13.3) times the mortality risk of patients with lower levels (adjusted p value = 0.007). Additional multivariable analyses indicate that lower stage (HR: 5.0; 95% CI: 1.7, 15.0; p = 0.004) and OPN level from the R&D kit less than the cutpoint of 212.6 ng/ml (HR: 3.5; 95% CI: 1.5, 8.4; p= 0.004) were significantly associated with improved survival.

In collaboration with the EDRN Data Management and Coordination Center, a blinded validation trial at three sites to establish ranges for measuring SMRP and OPN has been completed with over 680 sera using cohorts from Karmanos, NCI, Libby Montana, Australia, and Mt. Sinai Hospital of NYC, validation at three sites. Pending the results of this validation, a prospective trial examining these markers in the sera of villagers in the epidemic sites for MM in Cappadochia Turkey will be performed. Preliminary data from these villages regarding the sensitivity and specificity of SMRP and OPN are gratifyingly consistent with the data from the EDRN Biomarker Discovery Laboratory.

Keywords: mesothelioma, biomarkers, serum

467 Anti-MUC1 Antibody Levels and Risk for Ovarian Cancer in the Nurses' Health Studies

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Background: Antibodies against human mucin (MUC) member, MUC1 are found in individuals with various cancers, including breast and ovarian, and may predict better survival. Anti-MUC1 antibodies also can be found in women during pregnancy and lactation, leading to the hypothesis that a natural immunity against tumor MUC1 might account for the protective effect of pregnancy or breastfeeding on breast cancer risk. Based on case-control data, we previously concluded that some risk factors for ovarian cancer, including tubal ligation and ovulatory cycles, might be explained by their ability to raise or lower MUC1 immunity in the context of inflammatory or hormonal processes associated with those events in various tissues expressing MUC1. Our previous study was limited in that no pre-diagnostic bloods were available in cases. To assess the predictive value of anti-MUC1 antibody levels directly, we sought prospective specimens from the Nurses' Health Studies (NHS).

Methods: Plasma specimens from 117 cases at least three years prior to the diagnosis of ovarian cancer and from 339 age-matched healthy controls were assayed for total (IgM, IgG, and IgA) antibodies against a synthetic 100-mer MUC1 peptide corresponding to the five tandem repeats of the MUC1 polypeptide core tandem repeat region. We used generalized linear models to assess factors predicting antibodies and conditional logistic repression models to estimate risk associated with antibody levels.

Results: On average, subjects were age 57 at time of blood draw and cases were 65 at diagnosis. Among controls, antibody levels sharply declined after age 64 (p-trend=0.01), decreased with increasing number of estimated ovulatory months (p-trend=0.02), and increased with history of tubal ligation (p=0.03). Among cases, antibody levels non-significantly increased with age with the large majority of older cases having had high levels 5 or more years prior to cancer diagnosis. Compared to subjects with anti-MUC1 antibody levels below the first quartile, levels above the first quartile were associated with decreased risk for ovarian cancer of borderline significance, RR (95% CI) =0.65 (0.41-1.02) (p = 0.06); however, risk varied by age (p-interaction=0.009) (Table below).

Relative risks associated with Quartiles of anti-MUC1 antibody levels (measured by absorbance) in NHS specimens

	Q_1	Q_2	Q_3	Q_4	Q_1 vs. $Q_{2,3,4}$	p-trend *
	N (%)	*N (%)*	*N (%)*	*N (%)*	*N (%)*	
Age < 64 ‡						
Cases	32 (36)	23 (26)	17 (19)	18 (20)	58 (64)	
Controls	53 (20)	67 (26)	65 (25)	74 (29)	206 (80)	
RR (95% CI) †	1.00	0.57 (0.29, 1.09)	0.45 (0.22, 0.90)	0.43 (0.22, 0.83)	0.48 (0.28, 0.81)	*0.01*
Age ≥ 64 ‡						
Cases	8 (30)	4 (15)	6 (22)	9 (33)	19 (70)	
Controls	32 (40)	18 (23)	18 (23)	12 (15)	48 (60)	
RR (95% CI) †	1.00	0.86 (0.23, 3.25)	1.48 (0.42, 5.29)	3.44 (0.99, 11.9)	1.61 (0.62, 4.20)	*0.06*

Conclusion: Based on prediagnostic bloods from the NHS, anti-MUC1 antibody levels predict lower risk for ovarian cancer in women, especially those <64. Tubal ligation, known to decrease ovarian cancer risk, correlated with higher antibody levels; and ovulatory cycles, known to increase risk, correlated with lower levels. However, there appears to be a subset of women 64 or older who may have had chronically elevated anti-MUC1 antibodies and higher risk for ovarian cancer.

Keywords: ovarian neoplasms, MUC1, immunoglobulins

468 Centralized Biorepositories for Cooperative Group Clinical Trials: The North Central Cancer Treatment Group (NCCTG) Approach

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Purpose: The North Central Cancer Treatment Group (NCCTG) Biospecimen Resource was established to provide high quality biospecimens in support of translational research in the setting of prospective phase II and phase III clinical trials and to develop novel approaches to biospecimen procurement and analysis.

Methods: The NCCTG Biospecimen Resource, located at Mayo Clinic in Rochester, MN, includes paraffin-embedded tissue [tissue blocks, slides and tissue microarrays (TMAs)] and non-paraffin-embedded tissues including blood products [e.g., whole blood, plasma, serum, leukocytes, DNA, circulating tumor cells (CTCs)], urine, buccal cells, ascites, and frozen tumor tissue. The majority of NCCTG clinical trials utilize protocol-specific biospecimen collection kits that contain supplies, detailed instructions, and FedEx shipping labels to ensure correct biospecimen handling and to increase site compliance. Standard operating procedures and working instructions for various aspects of specimen handling include: biospecimen accessioning/tracking, sample processing (e.g., obtaining serum, plasma, DNA, and CTCs from blood, isolating nucleic acids from tissue, and constructing TMAs), specimen quality assessment, and biospecimen storage. All specimens are stored in appropriate restricted-access storage equipment for maximum preservation of the biological materials. Continuous temperature monitoring and appropriate alarm systems are employed on all storage refrigerators and low-temperature storage units. Prior to distribution for translational studies, biospecimens are assessed for the appropriate characteristics needed for the particular request. NCCTG is currently implementing a prospective pathology review which will be performed at the time of tissue receipt, which will include confirmation of diagnosis and recording of key histological characteristics such as size of tumor, percent invasive component, etc.

Results: NCCTG typically receives biospecimens from 70 to 90% of patients from whom they are requested. From 134 clinical trials to date, NCCTG has collected approximately 14,800 tumor tissue blocks from more than 8500 patients and over 26,600 body fluid biospecimens from more than 7000 patients (including ~1000 CTC samples from over 350 patients). Since 2006, roughly 11,000 paraffin-embedded tissue biospecimens and over 9000 body fluid biospecimens have been distributed to 43 investigators for translational research. Since 2000, 121 manuscripts utilizing NCCTG biospecimens have been published, demonstrating that specimens representative of clinical trial participants are available in sufficient numbers to answer the scientific questions posed in correlative laboratory studies.

Conclusion: The majority of NCCTG protocols currently accruing patients include biospecimen collection for protocol-defined translational studies and/or biospecimen banking for future translational studies such as biomarker immunoassays, proteomics, genotyping, mutation screening, immunohistochemistry, and/or gene expression profiling. NCCTG has demonstrated that high quality biospecimens can be collected successfully from community sites in multicenter Cooperative Group trials. Uniformity of biospecimen handling and access to complete clinical data create a powerful platform for translational research studies. Development of new methods for biospecimen collection, such as CTCs, provides promise for more robust translational studies in the future.

Keywords: biorepository, biospecimen, translational

469 HE4 has Strong Translational Potential as an Early Detection Marker for Ovarian Cancer

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Objective: A major goal of the Ovarian SPORE in Seattle has been to identify and evaluate in retrospective and prospective validation studies a screening test for epithelial ovarian cancer that employs one or more novel markers that complement CA125.

Background: We were the first [1] to report that the human epididymis 4 gene HE4, also known as WFDC2, is over-expressed in ovarian cancer. We developed the first [2] and additional [3, 4] assays to measure HE4 in serum, demonstrating that HE4 is a good diagnostic marker with better specificity than CA125. Others have confirmed these findings, reporting that HE4 performed best among 9 serum markers including CA125 [5]. In order to validate HE4 in preclinical serum sets, we needed a specimen-efficient assay. We therefore developed an HE4 assay using *in vivo* biotinylated recombinant antibodies of high affinity derived from clones or pools of anti-HE4-specific yeast display scFv [6]. Using this assay in a high throughput bead-based format we confirmed results of previous studies, showing that a panel combining HE4 with CA125 performs better than either marker used alone, that HE4 is a particularly good marker for serous and endometrioid cancer [7], and that in high-risk post-menopausal women, HE4 increases with age but is otherwise unaffected by population characteristics [8].

Methods: We are currently validating HE4 in inter-institutional and preclinical serum sets. Here we report results from a large set of clinical samples collected by the four Ovarian SPORE institutions for the first phase of a collaborative study with the Prostate, Lung, Colon and Ovary (PLCO) Cancer Screening Trial and the Early Detection Research Network (EDRN). In this study, 50 candidate markers were evaluated by laboratories at 4 institutions. In addition, we report the results from validation using preclinical samples obtained 0-2 years prior to diagnosis in the CARET trial.

Results: In all serum marker validation studies, HE4 has been consistently shown to be the best predictor of ovarian cancer other than CA125.

Conclusions: HE4 is a very promising diagnostic marker that is likely to complement CA125 in a screening program due to its better specificity.

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Keywords: ovarian cancer, early detection, HE4

470 Serum Fatty Acid Synthase as a Marker of Pancreatic Neoplasia

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Early detection markers of pancreatic cancer are needed to improve the uniformly poor prognosis of this disease. Fatty acid synthase (FAS), a metabolic enzyme that catalyzes the synthesis of long-chain fatty acids, is overexpressed in most human solid tumors and elevated FAS protein levels can be detected in the circulation of some cancer patients. We therefore evaluated FAS as a marker of pancreatic adenocarcinoma. FAS expression patterns in primary pancreatic adenocarcinomas, intraductal papillary mucinous neoplasms (IPMNs), and chronic pancreatitis tissues were analyzed using immunohistochemical analysis. Serum FAS levels were determined by ELISA in 102 patients with pancreatic adenocarcinomas, in 42 patients with IPMNs, in 27 patients with chronic pancreatitis, and in 39 healthy control subjects. For 11 patients with pancreatic adenocarcinoma, we determined FAS levels in both preoperative and postoperative serum samples. FAS protein was overexpressed in the ductal epithelium of 343/399 primary pancreatic adenocarcinomas (86%) and 28/30 IPMNs (93.3%), and in the islet and ductal cells in 3/54 chronic pancreatitis tissues (5/6%), whereas normal ductal epithelium lacked FAS expression. Serum FAS levels were significantly higher in patients with pancreatic ductal adenocarcinoma (mean +/- SD, 36.3 +/- 49.6 pg/ml), in patients with IPMNs (44.2 +/- 44.8 pg/ml), and in patients with chronic pancreatitis (41.0 +/- 50.7 pg/ml) than in healthy controls (2.7 +/- 6.0 pg/ml). Of 11 pancreatic adenocarcinoma patients for which we measured preoperative serum FAS levels, 8 (72.7%) had decreased FAS levels in their postoperative serum. These data suggest that overexpression of FAS in pancreatic cancer and its precursor lesions may contribute to elevated serum FAS levels in patients with pancreatic disease. Serum FAS measurement may help in the noninvasive diagnosis of pancreatic cancer and its precursors.

Keywords: pancreatic cancer, fatty acid synthase, intraductal papillary mucinous neoplasm

471 Genetic and Epigenetic Events in Papillary Thyroid Cancer and Their Potential for Clinical Translation

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Papillary thyroid cancer (PTC) is the most common endocrine malignancy that currently has a rapidly rising incidence, with an annual incidence of > 30,000 cases and a prevalence of > 300,000 cases in the USA. Although PTC is generally highly treatable, it can be aggressive in many patients with increased morbidity and mortality. The major medical treatment for PTC is radioiodine ablation therapy and morbidity and mortality associated with PTC are high in cases that have lost radioiodine avidity. A major underlying molecular mechanism is the aberrant silencing of thyroid iodide-handling genes that are responsible for iodide uptake and accumulation in thyroid cells. This represents a major therapeutic challenge for PTC. There are also several major diagnostic and prognostic obstacles associated with thyroid cancer and thyroid nodule due to the lack of effective diagnostic molecular markers. As *BRAF* mutation-promoted over-activation of the MAP kinase pathway plays a fundamental role in PTC tumorigenesis, we have proposed that thyroid gene silencing can be a consequence of *BRAF* mutation and MAP kinase pathway over-activation. We have also proposed that epigenetic alterations, particularly aberrant methylation, of these genes mediate the action of the MAP kinase pathway and these genetic and epigenetic patterns may be different in different types of thyroid cancer that are known to be associated with different clinical outcomes. A major research task of our laboratory has thus been to advance the understanding of the molecular processes involved in the interplay between genetic and epigenetic alterations in relation to the *BRAF* mutation and MAP kinase signaling, define their specific pathological roles, and establish molecular bases for the establishment of novel therapeutic targets and diagnostic molecular markers in PTC. To achieve this goal, we have been working on the following four Specific Aims: 1) Examine the common MAP kinase pathway-activating oncogenic events in PTC; 2) Examine the relationship between the major oncogenic events and the aberrant methylation of thyroid-specific genes in the common subtypes of PTC; 3) Examine the effects of alteration of the MAP kinase pathway activity on methylation and expression of thyroid-specific genes in thyroid tumor cell lines; and 4) Examine the diagnostic, prognostic, and therapeutic potential of these genetic and epigenetic events in PTC. We have made major efforts and achieved significant progress in achieving these goals as reflected by our many publications in recent years. This particularly includes our characterization of various genetic and epigenetic alterations as novel diagnostic and prognostic molecular markers and therapeutic targets for thyroid cancer. Examples include our documentation of *BRAF* mutation as a novel effective prognostic marker to guide risk stratification of PTC, including its novel use on diagnostic thyroid cancer needle biopsy specimens, characterization of serum methylation markers to diagnose primary thyroid cancer and monitor occurrence of PTC, and the testing of molecular abnormalities as therapeutic targets in the MAP kinase and PI3K/Akt signaling pathways (e.g., re-expression of thyroid iodide-handling genes and restoration of radioiodine uptake of thyroid cancer cells).

Keywords: thyroid cancer, molecular marker, prognosis

472 Aging, DNA Hypomethylation, Genetic Alterations, and Gastrointestinal Cancer.

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We investigated the frequency and extent of somatic DNA hypermethylation and hypomethylation in specific loci and genome wide to elucidate the chronology in gastrointestinal cancer of these epigenetic alterations and their potential application for early diagnosis and cancer susceptibility.

DNA methylation in specific sequences was comparatively analyzed by various techniques in a panel of over 200 gastrointestinal (colorectal, pancreatic and gastric) and ovarian cancers and cell lines. Global DNA methylation was also analyzed in gastritis and gastric cancer patients. We detected both hyper and hypomethylation alterations in some loci, either specific or common for these cancers.

An example of a gene commonly altered in different types of tumours is ADAMTS19, a member of the ADAMTS matrix metalloproteinase family. We found the promoter region of ADAMTS19 to be frequently hypermethylated in gastrointestinal tumours (26-43%) but less frequently in ovarian cancers (8%). However, ADAMTS19 hypermethylation in ovarian cancer was strongly associated to the mucinous phenotype: 9 out of 12 (75%) mucinous versus 1 out of 117 (0.9%) non-mucinous ($P < 10^{-9}$).

The association between ADAMTS19 hypermethylation and the mucinous phenotype in ovarian tumors could lead to a potential diagnostic biomarker for mucinous ovarian cancer.

In a previous study we estimated DNA methylation changes by MS-AFLP in colorectal and gastric cancer. Epigenetic damage, especially hypomethylation, associated with genomic damage and increased with age.

A band exhibiting greater demethylation in the MS-AFLP fingerprints was identified as a pericentromeric DNA repetitive element named SST1. Bisulfite sequencing showed severe demethylation of SST1 elements in about 20% of the primary tumors and 17% of cell lines.

Significant global demethylation was observed in gastric and colorectal cancer tissue relative to the normal adjacent tissues. This was generalized to patients from USA, Europe and Asia. Relative hypomethylation compared to healthy controls was also found in the normal gastric tissues from cancer and gastritis patients, but not in their relatives.

Severe demethylation of SST1 pericentromeric elements in human cancers may reflect changes in chromatin structure that ultimately may affect chromosomal integrity. The decrease in global levels of DNA methylation in normal tissues could also serve as a biomarker for gastrointestinal cancer susceptibility.

Keywords: colon cancer, genetics, epigenetics

473 Non-Invasive Detection of Candidate Molecular Biomarkers in Patients at High Risk for Colorectal Adenoma Recurrence

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Early detection of colorectal cancer can result in a high cure rate, therefore, an accurate screening method is imperative. Consistent with this goal, adoption of noninvasive methodology designed to reduce anxiety over colorectal cancer screening and improve overall acceptance of the screening process would be highly desirable. Unfortunately, current noninvasive detection methods lack sensitivity and will not detect alterations in gene expression. This is significant because changes in gene expression can modulate the regulatory mechanisms which either promote or protect against the development of colon cancer. Therefore, we have developed novel molecular methodology utilizing a stool sample, which contains intact sloughed colon cells, in order to quantify colonic gene expression profiles.

The effects of a legume enriched, low glycemic index, high fermentable fiber diet, were evaluated in participants with four possible combinations of risk factors, including insulin resistance (IR) and a history of adenomatous polyps. In a randomized crossover design controlled feeding study each participant consumed the experimental diet (1.5 cups of cooked dry beans) and a control diet (isocaloric average American diet) for 4 weeks with a 3 week washout period between diets. A total of 68 male subjects each of the four groups (17 each): 1. previous history of adenomas and IR; 2. previous history of adenomas without IR; 3. IR with no history of adenomas; and 4. non-IR and no history of adenomas were recruited. Investigation of the effects of patient risk and diet on global gene expression profiling was conducted using exfoliated cells.

We are investigating the potential to use gene expression profiles to discriminate between different phenotypes. The first goal, and the one considered to date, is to find genes that can discriminate between phenotypes (+IR, +Polyps) and (-IR, -Polyps) using the initial fecal data. We have concentrated on a single time point because using expressions across time-series data has proven to be confounding. Owing to the small sample sizes, it is expected that performance is limited and error estimation is problematic. Our goal is to show potential. Early results are encouraging. Based upon using prior biological knowledge we have greatly reduced the complexity of the feature selection problem to the point where we have been able to do an exhaustive search on all allowable feature (gene) sets of size 4, and among these, 27 have (unbiased) estimates error of 0.15 or less.

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Keywords: chemoprevention, biomarkers, non-invasive

474 Celecoxib Modulation of Lipoxygenase Pathways in Colonic Mucosa of FAP Patients

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Discovery of NSAIDs' crucial chemopreventive mechanisms will facilitate identification of new molecular targets and better chemopreventive agents. Lipoxygenase (LOX) pathways, including 5-, 12-, and 15-LOX-1 and -2, have been proposed to influence carcinogenesis in opposing directions (Shureiqi and Lippman, Cancer Res 2001). We have found in previous studies that (a) 13-hydroxyoctadecadienoic acid (13-HODE), a product of 15-LOX-1 metabolism of linoleic acid, are downregulated in human colorectal polyps and cancers, and 13-HODE restores apoptosis in colorectal cancer cells; and (b) celecoxib restoration of 15-LOX-1 expression triggers apoptosis in human colorectal cancer cells in vitro and is essential to inhibition of colonic tumorigenesis in human colorectal cancer xenograft mouse models. However, the corresponding effects of celecoxib on LOX pathways remain to be defined.

To determine the relevance of LOX modulation to celecoxib's chemopreventive effects, we tested the effects of celecoxib on 5-, 12-, and 15-LOX-1 and 15-LOX-2 products (LTB4, 12-HETE, 15-HETE, 13-HODE) in paired normal and polyp colonic mucosa biopsy samples obtained from patients with familial adenomatous polyposis (FAP) before (baseline) and after 6 months of celecoxib (400 mg orally twice daily). LOX products were measured using a sensitive and specific liquid chromatography/tandem mass spectroscopy method to simultaneously measure the HETE and HODE products. Measurements have been completed for 15 subjects. Baseline LTB4 levels were undetectable in 9 patients and minimally above the detection threshold for the other 6 (mean: 1.3 ng/mg protein); in all 15, LTB4 levels after celecoxib remained below or minimally above detection threshold. Baseline levels for the other LOX products were as follows:

	13-HODE		15-HETE		12-HETE	
Level of Product, ng/mg	Normal	Polyp	Normal	Polyp	Normal	Polyp
Mean	36.3722	25.64321	7.297396	6.501809	3.060838	2.49678
Standard error	4.824759	3.423975	0.748342	0.974779	0.747422	0.391884

Numbers of patients in which the LOX product level was higher in normal than polyp samples were: 13-HODE, 11; 15-HETE, 7; 12-HETE, 5. Numbers of patients with >50% changes in LOX product level after celecoxib were as follows:

Change Compared to Baseline	13-HODE	15-HETE	12-HETE
Increase	6	4	4
Decrease	2	3	3
None	7	8	8

In summary, 12- and 15-LOX-1 and 15-LOX-2 pathway products are detectable in colonic mucosa of FAP patients before and after celecoxib treatment. 13-HODE is the predominant of these products, and its levels are reduced in colonic polyps. Our results suggest that celecoxib effects differ by LOX pathway and patient. We are currently studying the relationship between the differential modulation of LOX products by celecoxib and clinical response.

Keywords: lipoxygenase, celecoxib, colonic polyps

475 Molecular Targets in Upper GI Cancer

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Barrett's esophagus (BE) is a major risk factor for the development of Barrett's-related adenocarcinoma (BAC) in the lower esophagus and gastroesophageal junction. The molecular alterations that lead to the development of BAC are poorly understood. In order to determine the possible diagnostic and therapeutic molecular targets in BAC, we have applied a comprehensive and systematic analysis of BAC using genomic, transcriptomic, proteomic, and epigenomic approaches. Our analysis revealed several important genomic amplifications with minimal common overlapping regions that included 1q21, 8q24, 17q12-q21, and 20q13. Using serial analysis of gene expression with sequence analyses of 20,000 clones produced a total of 457,894 expressed tags, 67,200 were unique. These tags represent 16,040 known gene symbols. Comparison of normal and tumor library results demonstrated an up-regulation of 242 genes and a down-regulation of 153 genes (two fold change, $p<0.05$). Integrated analysis of genomic amplification and gene expression revealed several oncogenomic transcriptional hot spots containing multiple genes of functional importance that show changes in expression, and may have clinical significance in tumorigenesis of BAC. Amplifications at 20q13 were observed in more than 50% of the cases. At this region we have identified several candidates, among which Aurora kinase A was the most promising. Further analysis confirmed amplification of AURKA in 30% of tumors and its over-expression at the mRNA and protein level in more than 50% of tumor samples that we studied. We have established the role of AURKA in tumorigenesis and demonstrated its role in cancer cell survival through activation of AKT and b-catenin signaling. The data indicated that AURKA could be a novel therapeutic target and through our collaboration with Millennium pharmaceuticals, we obtained and tested a recently developed AURKA specific inhibitor (MLN8054). The siRNA knockdown of AURKA, as well as the use of its pharmacologic inhibitor, has significantly reduced cancer cell survival *in vitro*. We have started the experiments to test the effect of MLN8054 as a single agent or in combined regimens *in vivo* using xenografted mouse models.

Using proteomic and epigenetic analysis approaches for analysis of BAC, we detected down-regulation of several genes that regulate the oxidative DNA damage. BACs develop on a background of chronic gastroesophageal reflux disease and increased levels of ROS. Therefore, we applied a systematic approach to analyze the promoter DNA methylation and gene expression of all members of the glutathione pathway. Quantitative anaylsis of DNA methylation using bisulfite pyrosequencing technology demonstrated a progressive increase in promoter DNA hypermethylation from BE to BACs with almost complete silencing of the expression of GPX3, GPX7, and GSTM2 in BACs. DNA is the most stable biological material as compared to RNA and protein. Therefore, we are directing our biomarker comprehensive analysis of DNA methylation in tumors, normal, and serum samples. We are utilizing state-of-the-art technologies using the methyl multiplex approach with Nimbelgen promoter arrays and the comprehensive ChIP-Seq approach using the Solexa whole genome sequencing technology.

Keywords: esophageal, cancer, genes

476 NSAIDs Modulate CDKN2A, TP53, and DNA Content Risk for Progression to Esophageal Adenocarcinoma (EA): A 10-Year Prospective, Single Center Cohort Study (EDRN Phase 4) for EA Risk Prediction

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Background: Somatic genetic CDKN2A, TP53, and DNA content abnormalities are common in many human cancers and their precursors, including esophageal adenocarcinoma and Barrett's esophagus (BE), conditions for which aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been proposed as possible chemopreventive agents; however, little is known about the ability of a biomarker panel to predict progression to cancer nor how NSAID use may modulate progression. We evaluated somatic genetic abnormalities and NSAID use as predictors of EA in a prospective cohort study of 243 patients with BE. **Methods and Findings:** Fresh frozen, flow sorted esophageal biopsies were evaluated at baseline for TP53 and CDKN2A (p16) alterations, tetraploidy, and aneuploidy using sequencing, loss of heterozygosity (LOH) by short tandem repeat (STR) polymorphisms, methylation-specific PCR, and DNA content flow cytometry. At 10 years, all abnormalities, except CDKN2A mutation and methylation, contributed to EA risk significantly by univariate analysis, ranging from 17p LOH (relative risk [RR] = 10.6; 95% confidence interval [CI] 5.2–21.3, p< 0.001) to 9p LOH (RR = 2.6; 95% CI 1.1–6.0, p = 0.03). A panel of abnormalities including 17p LOH, DNA content tetraploidy and aneuploidy, and 9p LOH was the best predictor of EA (RR = 38.7; 95% CI 10.8–138.5, p < 0.001). Patients with no baseline abnormality had a 12% 10-y cumulative EA incidence, whereas patients with 17p LOH, DNA content abnormalities, and 9p LOH had at least a 79.1% 10-y EA incidence. In patients with zero, one, two, or three baseline panel abnormalities, there was a significant trend toward EA risk reduction among NSAID users compared to nonusers (p=0.01). The strongest protective effect was seen in participants with multiple genetic abnormalities, with NSAID nonusers having an observed 10-y EA risk of 79%, compared to 30% for NSAID users (p < 0.001). **Conclusions:** A combination of 17p LOH, 9p LOH, and DNA content abnormalities provided better EA risk prediction than any single TP53, CDKN2A, or DNA content lesion alone. NSAIDs are associated with reduced EA risk, especially in patients with multiple high-risk molecular abnormalities. Subsequently, we completed analytic validation of a clinical grade, single plate SNP based platform using Pyrosequencing™ with universal PCR conditions that can assess 9p and 17p LOH within 2 hours and 10 minutes. The SNP panel was highly *specific* for 9p and 17p SNP genotypes. It had high *efficiency* with different input DNA quantities and improved genotyping calls in formalin fixed, paraffin embedded tissue compared to STRs. *Reproducibility* was 99% among 619 passing genotypes across varying PCR annealing temperatures. Highly robust genotypes were obtained down to 0.015ng DNA. In an experiment using FFPE samples ranging from 1ng (143 cells) to 0.06ng (<9 cells), the percentage of passing SNPs averaged 87% across all input DNA amounts. 92% of SNP LOH calls matched the STR LOH calls. We are also exploring SNP based assessment of LOH, copy number and aneuploidy using high density arrays. 91% of DNA content aneuploid populations could be distinguished from diploid populations by SNP array, and genome-wide measures of LOH or copy number accurately distinguished early and late stages of progression by ROC analysis (AUC = 0.91).**Next Research Extension:** Multicenter human studies of the biomarker panel (1) for EA risk prediction and (2) to direct randomized, double blinded aspirin chemoprevention trial.

Keywords: biomarkers, NSAIDS, Barrett's esophagus

477 Risk Assessment for Combination Chemoprevention for Recurrent Colon Polyps

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Chemoprevention strategies have not yet been implemented in standard medical practice for patients at high risk for developing colon cancer, due to limited efficacy and significant toxicity of candidate agents. We recently reported dramatic efficacy of combination chemoprevention with difluoromethylornithine (DFMO) and the non-steroidal anti-inflammatory drug (NSAID) sulindac to reduce recurrence of those colon polyps most highly associated with risk of colon cancer.

Combination chemoprevention with DFMO and sulindac was not associated with any serious toxicity, although our study population was small (375 patients). A sub-clinical ototoxicity was detected by quantitative audiology in a small subset of patients (less than 10%), and a not statistically significant trend was seen in cardiac toxicities in patients receiving treatment, compared to placebo. DFMO targets ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis. The *ODC* G315A single nucleotide polymorphism (SNP) affects *ODC* transcription and favorably modifies aspirin's effect on colorectal adenoma risk. In this study, we found that the ototoxicity was only statistically significantly associated with treatment in patients that were homozygous for the minor *ODC* A-allele (~5% of caucasians). Analysis of patient toxicities in trials of cyclooxygenase 2 (COX2) selective agents indicates that cardiotoxicites may be restricted to those patients with prior histories of these events.

These clinical data provide compelling evidence for efficacy of combination chemoprevention with DFMO and sulindac for prevention of recurrent colon polyps, especially those polyps associated with highest risk of progression to colon cancer. Additionally, these studies suggest that toxicities associated with combination DFMO and sulindac might be limited to subsets of patients that could be identified by either clinical history or simple blood tests assessing the *ODC* SNP. These findings suggest that combination chemoprevention might be safely and effectively applied in identifiable patients at risk for development of colon cancer.

Keywords: ODC, NSAIDS, chemoprevention, colon polyps, colon cancer, DFMO

478 Integrated Genomic Analysis of Esophageal Adenocarcinoma

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Esophageal adenocarcinoma incidence is increasing rapidly in the United States and in Western Europe. Surgery remains the best curative option for this tumor but almost 50% of patients are diagnosed with incurable metastatic disease. We have performed an integrated analysis of the esophageal adenocarcinoma genome and transcriptome in order to identify clinically relevant aberrations and potential therapeutic targets. We found that accumulation of DNA copy number alterations is an independent prognostic factor and used specific genomic regions to develop algorithms for survival prediction. Aberrant expression of individual genes within an altered region, such as reduced expression of the Huntingtin gene due to an apparent genomic loss, also correlated with poor survival. Both genomic alterations and subsequent gene expression changes determined lymph node metastasis with greater than 80% accuracy. Finally, we identified 90 genes where genomic amplification resulted in over expression. 10 of these genes are considered good potential targets for chemotherapy and/or biologic therapy. This is the first integrated genomic study in esophageal adenocarcinoma. The work presented defines genomic changes in this tumor and is an essential first step in the development of molecular staging and novel drug development.

Keywords: esophageal adenocarcinoma, genomics, DNA amplification

479 A Multicenter, Double-Blinded Prevalidation Study of Methylation Biomarkers for Progression Prediction in Barrett's Esophagus

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Background: Adenocarcinoma risk in Barrett's esophagus (BE) is increased 30-fold over the general population, but progression in BE is relatively rare. Biomarker-based prediction models would be useful in stratifying patients for more efficient surveillance endoscopy.

Methods: We performed a multi-center, double-blinded prevalidation study of a BE progression prediction model based on 8 methylation biomarkers. Progressors were categorized into two tiers: progression within 2 years (tier 1) or 4 years (tier 2). Methylation was assayed in 143 nonprogressors (NP) and 48 progressors (P) by real-time quantitative methylation-specific PCR.

Results: P were significantly older than NP (70.3 *vs.* 62.7 years, $p < 0.001$). We evaluated a linear combination of the 8 markers, using coefficients from a multivariate logistic regression analysis. Area under the ROC curve (AUC) was high in both the 2- and 4-year models (0.868 and 0.867; $p<0.001$ and $p<0.001$, respectively). Even after rigorous correction to eliminate potential overfitting, AUCs remained high and AUC shrinkage was minimal (AUC = 0.763 and 0.748; Δ-AUC = 0.105 and 0.119, respectively) in both models. We further explored the incremental AUC value of the 8-marker panel plus age *vs.* age alone. Even after correcting for overfitting, AUC values remained high (0.763 and 0.770, respectively), and incremental AUC values were substantial (Δ-AUC = 0.179 and 0.153, respectively) in both models. **Conclusions**: These findings suggest that a methylation biomarker-based strategy to predict Barrett's neoplastic progression is accurate and worthwhile.

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Keywords: Barrett's esophagus, progression, prediction, DNA methylation, dysplasia, esophageal adenocarcinoma

480 Diet, Inflammation and Tumor Formation in the Intestine

Klampfer, L., Kaler, P., Lin, E.Y., Deng, L., Li, J-F., Velcich, A., Tadesse, S., Guilmeau, S., Flandez, M., Wang, D., Newmark, H., Yang, K., Lipkin, M., **Augenlicht, L**

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A fully defined rodent diet formulated to mimic, both qualitatively and quantitatively, intake of major risk factors for colon cancer in the United States, accelerates and increases development of intestinal tumors in every mouse genetic model of intestinal cancer in which it has been investigated. As in the human population, this diet is effective in causing colon tumors when fed to wild-type, C57Bl/6 mice for approximately 2/3 of their lifespan (1.5-2 years). This is a model for sporadic colon cancer, responsible for >90% of colon cancer in western countries, and our data indicate this involves effects on gene expression profiles similar to those caused by inherited mutation of the Apc gene.

The risk factors in the western-style mouse diet are higher fat, lower calcium and vitamin D_3, and lower donors to the single carbon pool (folate, methionine, choline and fiber). The diet is formulated to adjust intake levels of these nutrients, on a nutrient-density basis, to reflect levels in the general population linked to elevated incidence of colon cancer. In both the genetic and dietary mouse models of intestinal cancer, elevating calcium and vitamin D_3 to levels demonstrated to be chemoprotective for human colon cancer prevents the increase in tumor formation, therefore identifying these nutrients as likely key components of the human diet that determine relative risk for colon cancer.

The development and progression of colon cancer is clearly associated with inflammation and can be significantly reduced by anti-inflammatory drugs. Since vitamin D_3 has both anti-inflammatory and chemopreventive activity, we investigated the role of inflammation and vitamin D_3 on tumorigenesis. Inflammation was generated in the intestinal mucosa using three mouse genetic models: in $Pofut^{flox/flox}$-villin:cre mice, which inactivates the fucosyltransferase necessary for efficient interaction of all Notch receptors with Delta and Jagged ligands, down regulation of Notch signaling led to pronounced secretory cell metaplasia in the small and large intestine, and to a major inflammatory response involving infiltration of macrophages, and T and B cells, associated with dysplasia that can progress to tumor formation (Guilmeau et al). Marcrophages and cells of the monocyte lineage, were more directly targeted by Lin and colleagues in generating a $Stat3^{flox/flox}$, cfm-cre model, which led to a rapid inflammatory response, and marked tumor formation; finally, Velcich uncovered a low-level inflammatory response in the $Muc2^{-/-}$ mouse she developed, in which the gene that encodes the major gastrointestinal mucin is inactivated and tumors arise throughout the small and large intestine, and rectum, that are increased by the western diet or by introduction of a mutant Apc allele. In each of these 3 models, there was also evidence for an important role of the intestinal microflora in the establishment of the inflammatory response.

Consistent with the particular importance of macrophages in tumorigenesis, Klampfer and colleagues have shown that conditioned media from human macrophage cultures, or co-culture of human macrophages with human colonic carcinoma cells, respectively, increased growth of the epithelial cells through stimulation of Wnt signaling in the epithelial cells. Moreover, elevation of growth and Wnt signaling in the epithelial cells was eliminated by treatment of the macrophages with vitamin D_3 that required expression of the vitamin D receptor by the macrophages, and was due to interleukin 1 signaling from the macrophages. In related work, Wang showed that colonic carcinoma cell growth could also be stimulated by mouse macrophages.

Thus, signals passing among and between mucosal inflammatory and epithelial cells, and the pathways involved, are potential targets for chemoprevention of colon cancer by dietary or pharmacological approaches.

Keywords: diet, inflammation, colon cancer

481 Blood-Based Detection of Colon Cancer Utilizing Specific Nuclear Matrix Proteins

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A blood test to detect colon cancer at a preventable stage would be a major advancement. We have previously identified colon cancer specific markers using focused proteomic analysis of nuclear structural proteins. Three of these markers, Colon Cancer Specific Antigen-2, -3 and -4 (CCSA-2, CCSA-3 and CCSA-4) have been developed into blood-based markers that are able to distinguish individuals with colorectal cancer from those without. Colon Cancer Specific Antigen-2 (CCSA-2) is a distinct novel colon cancer marker identified using focused proteomics.

Using an indirect enzyme-linked immunosorbent assay (ELISA) on serum samples obtained from two institutions, we evaluated CCSA-2 as a serum-based colon cancer marker. A total of 111 serum samples from individuals who underwent colonoscopy and were subsequently diagnosed as being normal (n=25) or having hyperplastic polyps (n=18), non-advanced adenomas (n=31), advanced adenomas (n=14) or colorectal cancer were evaluated (n=23). A diverse control population that consisted of 125 serum samples from individuals with other benign diseases and cancers was also included in this study.

Receiver Operating Characteristics (ROC) analyses were used to measure the sensitivity and specificity of CCSA-2. CCSA-2 at a cut-off of 10.8 μg/ml has overall specificity of 78.4% (95% CI 67.3 – 87.1%) and sensitivity of 97.3% (95% CI 85.8 – 99.5%) in separating individuals with advanced adenomas and colorectal cancer from normal, hyperplastic and non-advanced adenoma populations. The Receiver Operating Characteristics (ROC) curve for CCSA-2 has an area under the curve of 0.90 (95% CI 0.83 – 0.95).

Our initial study demonstrates that CCSA-2 is a potential serum-based marker for colon cancer detection with high sensitivity and specificity, as well as in detecting individuals with advanced adenomas. Larger scale and multi-institutional validations are necessary to further determine the clinical potential of this serum-based test as well as those developed for CCSA-3 and CCSA-4.

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Keywords: CCSA-2, colon cancer, early detection biomarker

482 Identification and Characterization of Amplified Gene Targets in Esophageal Adenocarcinoma

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Esophageal cancer remains a highly lethal malignancy and the incidence of its most prevalent histologic subtype, esophageal adenocarcinoma, has been increasing rapidly. Genomic DNA amplification occurs selectively in many cancers including esophageal adenocarcinoma, and we have identified a number of specific amplicons that may have a direct role in esophageal adenocarcinoma tumorigenesis. Genes isolated from these regions of amplification may provide novel targets for the early cancer identification or treatment. We utilized a large prospectively-collected tissue bank of fresh-frozen resected cancers with corresponding normal tissue, serum and clinical characteristics to identify potential amplification events in genomic DNA from selected cases using several discovery-based platforms including restriction landmark genome scanning, array CGH, and SNP arrays.

Recurrent gene amplification was detected at many loci including several which have not been previously detected in esophageal adenocarcinoma, or that definitive candidate genes have been described. Confirmation of several of the most frequently amplified novel regions was assessed at the DNA level using qPCR and also at the mRNA level by qRT-PCR to help identify candidate cancer genes. Further analysis using Affymetrix gene chip of Barrett's metaplasia, dysplasia and adenocarcinoma provide an assessment of the expression during disease progression. An example is our identification of a region on 18q11 which is amplified in 20% (18/87) of esophageal adenocarcinomas and is the second most frequently amplified locus after the *erbB2* gene (22%) in this cancer. This region was narrowed by elimination of flanking genes and shown to include only the genes for transcription factor *GATA6* and mind bomb 1 (*MIB1*). *GATA6* gene overexpression in amplified cases was confirmed with Affymetrix gene chips and qRT-PCR, and GATA6 protein tissue overexpression in amplified cases was confirmed with tissue microarrays-immunohistochemistry as well as with Western analyses. Ongoing functional studies are determining the effects of GATA6 overexpression in esophageal adenocarcinoma cell lines.

This and other amplified gene regions provide potential markers for early cancer detection, monitoring cancer recurrence, as well as targets for therapeutic intervention. Several of the most frequently amplified regions in esophageal adenocarcinoma include known targets for therapy (*erbB2, EGFR, Met*) indicating the potential for defining gene amplification events and selection of appropriate patient treatments.

Keywords: Barrett's adenocarcinoma, early detection, patient selection

483 The AGR2 Gene as a Target for Early Diagnosis and Therapeutic Intervention

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Barrett's esophagus is a premalignant lesion that increases the risk for esophageal adenocarcinoma. The AGR2 gene is highly expressed in Barrett's esophagus and esophageal adenocarcinoma. AGR2 is also expressed in a variety of other adenocarcinomas including colon, pancreatic, breast, and prostate cancers. Reduction of AGR2 expression in mouse tumor xenografts compromised growth. In addition, induced AGR2 expression in NIH3T3 cells results in foci formation and the capacity for anchorage independent growth in soft agar, which are both in vitro characteristics of transformation.

Studies of AGR2 expression in normal cells revealed that it is expressed in a distribution consistent with areas of cell proliferation in the intestinal crypts in the gastrointestinal tract. We have established that intestinal cells of secretory lineage specifically express AGR2. The results suggest that AGR2 may represent a link between gastrointestinal lineage-specific stem cells and cancer.

Current NIH funded efforts are focused on defining AGR2's mechanism of action. We will define the essential AGR2 protein domains required for its actions, the genomic domains and determinants for its expression, and the genes and proteins whose expression is affected by AGR2. It is likely that AGR2's effects are mediated via activation of signal transduction pathways, the identity of which will also be determined. In view of the biologic results obtained to date, AGR2 is a promising target for therapeutic intervention. Defining AGR2's mechanism of action will guide future strategies for therapeutic intervention. It is also likely that many other adenocarcinomas that express AGR2 will benefit from an understanding of this gene's biology.

In addition its role as a potential therapeutic target, AGR2 may serve as a valuable marker of disease. Our previous published work demonstrated high AGR2 expression in Barrett's esophagus and esophageal adenocarcinoma. Detection of AGR2 expression or the activation of associated signal transduction pathways represents a promising avenue for early diagnosis using either a conventional approach in pathology or emerging imaging technologies. Easy access to the esophagus through the oral cavity greatly enhances the delivery of potential diagnostic reagents.

Keywords: Barrett's esophagus, esophageal cancer, AGR

484 Aspirin, UGT1A6 Genotype and Colon Gene Expression: Results From an Aspirin Intervention Trial

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Regular use of aspirin and other non-steroidal anti-inflammatory drugs (NSAID) reduces the incidence of colon adenomas and carcinomas by approximately 50%. The UDP-glucuronosyltransferases (UGT) enzymes are prominently involved in metabolizing aspirin. *UGT1A6* is a polymorphic UGT and its variant alleles seem to metabolize aspirin less efficiently. In NSAID users, and particularly aspirin users, the risk for colon neoplasia is reduced only in individuals who are *UGT1A6* heterozygous and homozygous for the variant *2 allele, but not homozygous for the wild-type *1 allele. The aim of our study was to determine the effect of *UGT1A6* genotype on aspirin metabolism and aspirin-induced changes in colonic gene expression using an observational study and an aspirin intervention. Participants (n = 505) recruited from the Seattle area included healthy men and women, age (mean $\pm$ SD) 30.6 $\pm$ 7.3 years, with no history of gastrointestinal disease, 51% female, and 28.6% minorities. Participant buffy coat was genotyped for *UGT1A6* T181A and R184S polymorphisms and each participant completed an 8-h urine collection following aspirin dosing (650 mg) to measure aspirin metabolites. The genotype distributions were: 50% (*1/*1), 41% (*1/*2), and 9% (*2/*2). Although there were statistically significant sex differences in urinary excretion of aspirin and its metabolites (women excreting a higher percentage of salicylic acid glucuronides), there were no differences in metabolite excretion by UGT1A6 genotype [stratified by race and adjusted for urine volume, urine pH, energy and fruit and vegetable intake, body mass index, and dietary supplement use].

A subset of participants in the observational study were recruited into a randomized, placebo-controlled trial of aspirin supplementation. 22 *UGT1A6* *1/*1 individuals and 22 *UGT1A6* *2/*2 individuals were assigned to a daily dose of 325 mg aspirin or a placebo for 2 months in a randomized crossover design. There was at least a 3-month washout between treatment periods. Biopsies from the sigmoid colon and rectum were taken during sigmoidoscopy at the end of each 2-month treatment period. We assayed gene expression from both stromal and epithelial samples of sigmoid tissue using Affymetrix U133 2.0 microarrays. A comparison of stromal and epithelial gene expression revealed over 4800 genes differentially expressed between these two tissue types under either treatment. Pathways represented by genes expressed in epithelium included lipid metabolism and drug and xenobiotic metabolism, whereas inflammation, immune response and extracellular matrix were more represented in stroma. The Wnt pathway was represented in both compartments. Preliminary evaluation of the array data for aspirin effects suggests, at best, a modest impact of the intervention on gene expression in these samples. Principle component analysis revealed three components in the stromal compartment that partitioned with treatment at a p-value <0.1. There did not appear to be an effect of UGT1A6 genotype on gene expression. These results suggest that the reported modifying effects of the UGT1A6 genotype on the association between aspirin use and colorectal adenoma risk may not be due to differences in aspirin metabolism. Further, effects of a daily dose of 325mg aspirin on gene expression in normal colon tissue are modest and difficult to detect by a conventional array approach.

Keywords: UGT, aspirin, gene expression

485 Genetic Diversity and Clonal Expansions in the Neoplastic Progression of Barrett's Esophagus

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Neoplastic progression is a process of clonal evolution. We are developing measures of that evolutionary process for risk stratification in the pre-malignant condition of Barrett's esophagus (BE). We will use these biomarkers to measure the evolutionary mechanisms through which non-steroidal anti-inflammatory drugs (NSAIDs) and vitamin C may prevent progression in BE. We have previously shown that the amount of genetic diversity, as defined by loss of heterozygosity (LOH) in microsatellite loci and ploidy measured by DNA content, predict progression to esophageal adenocarcinoma. We have also shown that clones can expand to fill the BE segment and the size of clones with p53 LOH, aneuploidy and/or tetraploidy, predicts progression. We are now adapting these measures to SNP array platforms in a large (n=614) cohort of participants with BE. In addition, previous observations in this cohort have shown that NSAID use and vitamin C are associated with dramatic reductions in the risk of progression. We will measure the associations of NSAID and vitamin C supplement use on clonal genetic diversity and the incidence of large clonal expansions measured over time within participants. In addition, we are seeking to develop and test single cell genetic diversity assays. These will be important for generalizing diversity assays to neoplasms in which distinct clones cannot be identified by taking multiple biopsies. By assaying genetic differences between single cells, we will be able to measure diversity within single samples such blood, urine, feces, sputum, and potentially, single biopsies.

Keywords: clonal evolution, Barrett's esophagus, non-steroidal anti-inflammatory drugs (NSAIDs)

486 Novel Fucosylated Biomarkers for the Early Detection of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most common cancer, but the third leading cause of cancer death, with more than 500,000 fatalities annually. The high mortality associated with HCC is partially due to late diagnosis and poor response to treatment. We have previously reported changes in N-linked glycosylation that occur with the development of liver cancer and, through the use of glycoproteomics, identified over 50 proteins with altered glycosylation as a function of HCC. The altered glycosylation of many of these protein were analyzed individually, in combination with our previously identified biomarker, Golgi protein 73 (GP73) or in combination with the currently used marker, alpha-fetoprotein (AFP). This analysis was performed on two separate coded patient cohorts consisting of a total of 277 patients with cirrhosis or cirrhosis plus HCC. Individually, many of these markers had greater performance than AFP alone, however, greatest performance was achieved through the combination of two markers, fucosylated hemopexin and total GP73, which had an optimal sensitivity of 100%, a specificity of 75% and an AUROC of 0.96. In conclusion, the altered glycosylation of serum glycoproteins can act as potential biomarkers of primary hepatocellular carcinoma when used independently or in combination with other markers of liver cancer.

Keywords: hepatitis, hepatocellular carcinoma, glycosylation

487 The Interplay of Transformed Epithelial Cells and Stromal Fibroblasts in the Tumor Microenvironment

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The stratified squamous epithelium is the most common type of epithelium in humans. Proliferating basal cells, residing upon the basement membrane, migrate in an outward direction undergoing differentiation. Superficial cells desquamate by virtue of senescence and apoptosis. Epithelial renewal occurs in a tightly regulated fashion during normal homeostasis and during tissue regeneration. Using esophageal epithelial cells as a platform, we have found that they can be transformed genetically employing a combination of EGFR overexpression, hTERT activation and p53 mutation, all with stable gene expression with retroviral vectors. Three-dimensional culture (also referred to as organotypic culture or tissue reconstructs) confers intrinsic advantages of evaluation of the interplay between different cellular compartments and cell types, thereby mimicking the tumor microenvironment. As a result, we find that the age and source of fibroblasts influences tumor cell migration and invasion into the extracellular matrix (ECM) through biological and physical cues. In aggregate, stromagenesis influences tumorigenesis and this may unravel new opportunities to identify molecular pathways that promote tumor cell migration and invasion directed by activated stromal fibroblasts.

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Reference from Rustgi lab: Okawa T, Michaylira C, et al. *Genes & Development* 2007;21(22):2908-22.

Keywords: tumor microenvironment, stromal fibroblasts, oncogenes/tumor suppressor genes

488 Using Proteomics to Develop Chemoprevention Targets for Colon Cancer

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Genetic background, as well as environmental factors, are likely to influence the efficacy of intervention strategies for colon cancer prevention. One approach to optimizing cancer chemoprevention is to identify individuals that will be responsive to a particular agent. This could in principal be accomplished using genetic approaches, provided that genes important for a response may be identified. On the other hand, a proteomic approach offers a number of distinct advantages, including the ability to detect post-translational modifications of proteins that play a central role in generating a positive response.

In the following study, we describe the development of a proteomic-based approach to identifying tumor changes in real-time in response to sulindac treatment. It will be possible to determine which sub-populations of early colon lesions develop into tumors, and whether chemoprevention agents suppress the rate of adenoma formation, or promote their regression. Our method is predicted to recapitulate potential clinical scenarios, in which protein markers can be used to identify individuals with 'high-risk' adenomas. In addition, our long-term goal is to customize chemoprevention in human populations based on expression of predictive proteins or genes uncovered in precancerous lesions.

Our initial approach is to characterize the response to sulindac, a commonly used chemoprevention agent that has varying efficacy. If we are successful, this general strategy can be adapted to many other chemoprevention agents. In addition, we anticipate that our refinements in lesion imaging and feature recognition within the topography of the colon, garnered from mouse chromoendoscopy, will eventually be translated into the clinic as a procedure for monitoring the precise location of lesions that can then be followed longitudinally over time.

Reference: Nakanishi et al., Proteomics 1(12):1660-1666.

Keywords: colon cancer, chemoprevention, proteomics

489 Mechanisms of Acid Resistance in Barrett's Esophagus

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Barrett's esophagus is a term used to indicate the presence of specialized columnar epithelium (SCE) lining the distal esophagus. It develops in the setting of gastroesophageal reflux disease (GERD) as replacement for acid-destroyed esophageal stratified squamous epithelium. For obvious reasons, clinical attention has focused on the risk of esophageal adenocarcinoma in Barrett's esophagus, and yet Barrett's SCE remains stable for decades in the vast majority of subjects, and Barrett's subjects have a life expectancy similar to that of the general population. Moreover, stability is the norm irrespective of whether Barrett's is treated with acid suppressants such as proton pump inhibitors, since most with Barrett's, presumably because of their acid resistance, are (reflux) symptom-free. In one sense, then, Barrett's SCE can be viewed as a 'successful adaptation' for protection of the esophagus against further reflux damage. Nonetheless, the concept of SCE as a protective phenomenon has all but gone unnoticed and this despite the fact that acid resistance is at the very heart of its existence. For this reason, our laboratory has begun an examination of the mechanisms for acid resistance in Barrett's esophagus and contrasts them to those of native esophageal stratified squamous epithelium. The results of the studies to date indicate that Barrett's has a number of processes that enable it to better tolerate an acid insult. One process that is evident is through its greater capacity for surface buffering of backdiffusing luminal H^+ than that observed in squamous epithelium. This is in part due to the ability of Barrett's SCE to secrete both bicarbonate and mucus – and both of these abilities known to be lacking in esophageal squamous epithelium. Another protective process in Barrett's SCE resides in its barrier function against H+ and this attributed to possession within its tight junctions of claudin-18. The importance of claudin-18 - which is notably absent in the tight junctions of squamous epithelium – is that it has been shown to reduce the permeability of the paracellular pathway to H+. Since the tight junctions governing paracellular pathway in squamous epithelium are particularly vulnerable to attack and damage by luminal H+, conversion of the esophageal lining from one without claudin-18 (squamous) to one with claudin-18 (SCE) appears to be another means for protection against an acid insult. In effect, Barrett's SCE appears to have evolved a set of properties specifically designed for survival in GERD patients who harbor a lower esophageal environment acidic enough to have destroyed the native squamous epithelium.

Keywords: mucus, bicarbonate, tight junction

490 Molecular Detection of Occult Nodal Metastases in Esophageal Adenocarcinoma

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Introduction: Esophageal adenocarcinoma (EAC) is an aggressive malignancy whose incidence is on the rise. Approximately 40% of patients with N0 disease will recur after theoretically curative surgery, suggesting that in early stage disease, metastatic spread is often undetected by routine pathology. Molecular techniques may more accurately detect micrometastatic spread of EAC, but the correlation between molecular analysis of nodes and prognosis is unknown. Our lab has previously identified and validated 4 markers whose gene expression levels are able to distinguish benign nodes from nodes with metastatic EAC: CK19, CK20, CEA and TACSTD1. We used quantitative real-time RT-PCR to evaluate the expression of these 4 markers in lymph nodes from 68 N0 and 62 N1 EAC patients to see if molecular staging is predictive of a worse clinical outcome.

Methods: RNA was isolated from 1456 lymph nodes obtained from 130 patients who underwent resection of EAC. QRT-PCR was used to analyze gene expression for each of the 4 markers. Relative expression of each marker was compared with expression in 53 benign esophageal lymph nodes previously analyzed.

Results: Analysis of 778 lymph nodes from 68 pN0 patients identified 71 nodes (9%) from 30 patients (44%) which showed positive expression of at least one marker, indicating occult metastases (and molecular upstaging). Analysis of 678 lymph nodes from 62 pN1 patients revealed 141 nodes (21%) from 40 patients (65%) which had positive expression of at least one marker in nodes that were pathologically negative. In the pathologically positive nodes from N1 patients, there was an encouraging 88% concordance between pathological and molecular analysis. After a median follow-up of 2 years, 13 N0 patients had recurrence of their cancer. Patients who were node negative by routine pathological examination but who were molecularly positive with increased in gene expression levels of 3 of the 4 markers (CK20, CEA and TACSTD1) in the lymph nodes experienced a significantly worse disease-free and overall survival (p values <0.05).

Conclusion: Molecular positivity determined by increased gene expression of markers in patients who are node negative by routine pathology examination, is predictive of significantly worse disease-free and overall survival in EAC patients. These results will lead to Phase II trials, which are currently being designed investigating adjuvant therapies in high risk patients with esophageal cancer.

This work was supported by NIH grant 2 RO1 CA090665-05.

Keywords: esophageal cancer, molecular staging, prognostic evaluation

491 Exploiting the Genome and Transcriptome for Individualized Cancer Diagnosis and Treatment Stratification

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Genetic Markers for Disease Progression of Cervical Dysplasia

Invasive cervical carcinomas almost invariably carry extra copies of chromosome arm 3q, resulting in a gain of the human telomerase gene (*TERC*). We therefore decided to explore whether gain of 3q and genomic amplification of *TERC* can predict progression from CIN1 and CIN2 to CIN3 and invasive carcinoma. We applied FISH with a triple-color fluorescent probe set to a series of 59 previously stained Pap smears for which repeat Pap smears and clinical follow-up were available. The samples included (1) CIN1 and CIN2 lesions that progressed to CIN3, (2) CIN1 and CIN2 lesions that regressed spontaneously, and (3) normal Pap smears from women who subsequently developed CIN3 or cervical cancer. We now show that CIN1/CIN2 lesions that progress to CIN3 lesions or cancer revealed a gain of 3q. Our data therefore proof that 3q gain is required for the transition from CIN1 and CIN2 to CIN3 and that it predicts progression. None of the spontaneously regressing CIN1/CIN2 lesions showed this genetic aberration. Of note, 3q gain was found in 33% of cytologically normal Pap smears from women who were diagnosed with CIN3 or invasive cervical carcinoma after a short latency.

Response Prediction of Patients With Rectal Cancer Treated With Radiochemotherapy

There is a wide spectrum of tumor responsiveness of rectal adenocarcinomas to preoperative chemoradiotherapy ranging from complete response to resistance. We therefore investigated whether gene expression profiling can assist in stratifying patients into responders or non-responders. Pretherapeutic biopsies from 30 patients with locally advanced rectal adenocarcinomas were analyzed using microarrays. Class comparison was used to identify genes that were differentially expressed between responders and non-responders. Responders and non-responders showed significantly different expression levels for 54 genes. When we applied LOOCV to predict response to therapy, we were able to correctly predict the tumor behavior in 83% of patients. Our results suggest that pretherapeutical gene expression profiling may assist in response prediction of rectal adenocarcinomas to preoperative chemoradiotherapy and in prediction of disease free survival if validated in larger independent studies.

Keywords: cervical cancer, rectal cancer, genomics, aneuploidy

492 Array-Based Comparative Genomic Hybridization Reveals Potential Tumor Driver Genes in Hepatocellular Carcinoma With Stem/Progenitor Cell-Like Phenotype

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Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide. HCC is very heterogeneous in terms of its clinical presentation as well as genomic and transcriptomic patterns. The discovery of genomic changes in other cancer types has led to the development of new specific therapies, underscoring the importance of understanding molecular mechanisms and tumor biology to guide personalized therapy. To this end, we performed mRNA gene expression analysis of HCC and identified four HCC subtypes, one of which (hepatic stem cell-like HCC; HpSC-HCC) displayed features of stem/progenitor cell origin with poor survival outcome. In contrast, clinical HCC specimens resembling mature hepatocytes (MH-HCC) were associated with good survival outcome. We hypothesized that particular HCC subtypes with unique gene profiles may evolve from the disruption of certain genetic loci which may be critical to HCC development. We therefore performed array-based comparative genomic hybridization (arrayCGH) at a 15 KB resolution to compare the genomic DNA of clinical HCC specimens (N=76). We found that HpSC-HCC tumors appear to differ greatly from MH-HCC. Grossly, gain of 1q21-31 and loss of 4q26-35 occur mainly in HpSC-HCC whereas gain of 17q22-24 exclusively occurs in MH-HCC. In total, 848 genes displayed significant differences in the frequency of copy number changes (frequency difference>20%, $p<0.05$). To identify potential cancer driving genes we compared the arrayCGH and mRNA expression data. The distribution of the resulting Pearson correlation coefficients displayed a shift to positive correlation (10,841 genes, mean $r=0.185$, $p<0.001$). 126 of the 848 genes identified by arrayCGH were correlated with the mRNA expression status ($r>0.3$). This 126-gene signature was used on mRNA expression arrays for class prediction in an independent set of 73 MH- and HpSC-HCC samples. Seven class prediction tools were able to predict these independent samples with 70 to 83% accuracy (10-fold cross-validation, $p<0.001$). These findings reveal that HpSC-HCC and MH-HCC are genetically distinct tumor subtypes which may coincide with HCC development. To further analyze this 126-gene signature, we are currently performing pathway analysis and gene set enrichment to identify genes as potential targets for subtype specific therapy.

Keywords: EpCAM, tumor subtypes, arrayCGH

493 MicroRNA Expression Patterns Are Associated With Prognosis And Therapeutic Outcome In Colon Adenocarcinoma

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Context: MicroRNAs have potential as diagnostic biomarkers and therapeutic targets in cancer. No study has evaluated the association between microRNA expression patterns and colon cancer prognosis or therapeutic outcome.

Objective: To identify microRNA expression patterns associated with colon adenocarcinomas, prognosis, or therapeutic outcome.

Design, Setting, and Patients MicroRNA microarray expression profiling of tumors and paired nontumorous tissues was performed on a US test cohort of 84 patients with incident colon adenocarcinoma, recruited between 1993 and 2002. We evaluated associations with tumor status, TNM staging, survival prognosis, and response to adjuvant chemotherapy. Associations were validated in a second, independent Chinese cohort of 113 patients recruited between 1991 and 2000, using quantitative reverse transcription polymerase chain reaction assays. The final date of follow-up was December 31, 2005, for the Maryland cohort and August 16, 2004, for the Hong Kong cohort.

Main Outcome Measures: MicroRNAs that were differentially expressed in tumors and microRNA expression patterns associated with survival using cancer-specific death as the end point.

Results: Thirty-seven microRNAs were differentially expressed in tumors from the test cohort. Selected for validation were *miR-20a*, *miR-21*, *miR-106a*, *miR-181b*, and *miR-203*, and all 5 were enriched in tumors from the validation cohort (*P*_.001). Higher *miR-21* expression was present in adenomas (*P* = .006) and in tumors with more advanced TNM staging (*P*_.001). In situ hybridization demonstrated *miR-21* to be expressed at high levels in colonic carcinoma cells. The 5-year cancer-specific survival rate was 57.5% for the Maryland cohort and was 49.5% for the Hong Kong cohort. High *miR-21* expression was associated with poor survival in both the training (hazard ratio, 2.5; 95% confidence interval, 1.2-5.2) and validation cohorts (hazard ratio, 2.4; 95% confidence interval, 1.4-3.9), independent of clinical covariates, including TNM staging, and was associated with a poor therapeutic outcome.

Conclusions: Expression patterns of microRNAs are systematically altered in colon adenocarcinomas. High *miR-21* expression is associated with poor survival and poor therapeutic outcome.

Keywords: microRNA, colon adenocarcinoma, prognosis

494 Over-Expression of CDC25B and LAMC2 mRNA and Protein in Esophageal Squamous Cell Carcinomas and Pre-Malignant Lesions in Subjects From a High-Risk Population in China

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Molecular events associated with the initiation and progression of esophageal squamous cell carcinoma (ESCC) remain poorly understood, but likely hold the key to effective early detection approaches for this almost invariably fatal cancer. *CDC25B* and *LAMC2* are two promising early detection candidates emerging from new molecular studies of ESCC. To further elucidate the role of these two genes in esophageal carcinogenesis, we performed a series of studies to: (i) confirm RNA over-expression; (ii) establish the prevalence of protein over-expression; (iii) relate protein over-expression to survival; and (iv) explore their potential as early detection biomarkers. Results of these studies indicated that *CDC25B* mRNA was over-expressed (≥2-fold over-expression in tumor compared to normal) in 64% of the 73 ESCC cases evaluated, while *LAMC2* mRNA was over-expressed in 89% of cases. CDC25B protein expression was categorized as positive in 59% (144/243) of ESCC cases on a tumor tissue microarray, and non-negative LAMC2 patterns of protein expression were observed in 82% (225/275) of cases. Multivariate-adjusted proportional hazard regression models showed no association between CDC25B protein expression score and risk of death (Hazard Ratio [HR] for each unit increase in expression score = 1.00, P=0.90), however, several of the LAMC2 protein expression patterns strongly predicted survival. Using the cytoplasmic pattern as the reference (the pattern with the lowest mortality), cases with a diffuse pattern had a 254% increased risk of death (HR=3.52, P=0.007), cases with no LAMC2 expression had a 169% increased risk of death (HR=2.69, P=0.009), and cases with a peripheral pattern had a 130% greater risk of death (HR=2.30, P=0.02). CDC25B protein expression scores in subjects with esophageal biopsies diagnosed as normal (n=35), dysplastic (n=23), or ESCC (n=32) increased significantly with morphologic progression. For LAMC2, all normal and dysplastic patients had a continuous pattern of protein expression, while all ESCCs showed alternative, non-continuous patterns. This series of studies showed that both CDC25B and LAMC2 over-express RNA and protein in a significant majority of ESCC cases. The strong relation of LAMC2 pattern of protein expression to survival suggests a role in prognosis, while CDC25B's association with morphologic progression indicates a potential role as an early detection marker.

Keywords: esophageal cancer, *CDC25B*, *LAMC2*

495 A Novel Rat "Patient-Like" Model of Intrahepatic Cholangiocarcinoma Progression Closely Mimicking Clinical and Molecular Features of the Human Disease

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Intrahepatic cholangiocarcinomas are relatively rare, but highly malignant hepatobiliary cancers that are typically diagnosed at an advanced stage, thus presenting a significant biomedical and therapeutic challenge. Development of preclinical models of cholangiocyte neoplastic transformation and malignant progression that closely recapitulate the molecular etiology and malignant behavior of the advanced clinical human disease would provide an important resource for investigating mechanisms regulating cholangiocarcinoma growth and progression. In addition, such models have a strong potential of serving as powerful preclinical platforms for testing novel molecular targeting strategies for cholangiocarcinoma therapy.

The aims of this study were to: (1) develop such a preclinical model, based on bile duct inoculation of a highly malignant oncogenic neu-transformed rat cholangiocyte cell line, designated as BDEneu (Gastroenterology 2005; 129: 2047-2057), versus a less aggressive spontaneously transformed rat cholangiocyte cell line designated as BDEsp (Hepatology 2008; 47: 1178-1190), into livers of isogenic Fisher 344 rats; (2) investigate the role of bile duct obstruction, a common serious complication of malignant biliary cancer, on cholangiocarcinoma progression; and (3) assess the therapeutic potential of the clinically relevant dual ErbB1/ErbB2 tyrosine kinase inhibitor, lapatinib, on the tumorigenic growth of orthotopically transplated BDEneu cells overexpressing activated $p185^{neu}$ (ErbB2) oncoprotein, together with up-regulated epidermal growth factor receptor (ErbB1). Tumorigenic BDEneu cells expressed significantly increased constitutive levels of $p185^{neu}$ phosphorylated at tyrosine 1248, phosphorylated p42/44 MAPK, and MUC1 mRNA (a marker of cholangiocarcinoma progression) than did BDEsp cells, whereas COX-2 and phospho-Akt levels were found to be more prominently expressed in BDEsp cells than in BDEneu transformants. BDEneu cells inoculated into the bile duct of isogenic rats exhibited over a three week period an exponential pattern of rapid tumor growth in liver, correlating with a progressive development of bile duct obstruction, reflected by significant increases in serum bilirubin levels. In this orthotopic hepatobiliary cancer model, bile duct obstruction induced by tumorigenic growth or by bile duct ligation was further demonstrated to be a potent stimulus for intrahepatic cholangiocarcinoma growth and progression, as well as of peritoneal carcinomatosis.

Under comparable conditions, BDEsp cells yielded only small nonmetastatic intrahepatic cholangiocarcinomas without gross pathological evidence of bile duct obstruction (i.e., an icteric liver). Rats administered lapatinib by gavage at a dose of 75 mg/kg, twice a day, beginning two days after bile duct inoculation of BDEneu cells and continuing for an additional 23 days, showed a 68% reduction in mean liver tumor wet weight and a 72% decrease in mean serum bilirubin level compared with vehicle-treated controls. In contrast, lapatinib was without effect in inhibiting intrahepatic tumor growth and jaundice when its administration was delayed. These results support a novel model of intrahepatic cholangiocarcinoma progression that appears to be well suited for preclinical testing.

Keywords: neu-transformed rat cholangiocytes, orthotopic cell transplantation, ErbB- targeted therapy

496 GUCY2C in Lymph Nodes Predicts Time to Recurrence and Disease-Free Survival in pN0 Colorectal Cancer

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Of patients with node-negative (pN0) colorectal cancer, about 25% develop recurrence after surgery. GUCY2C is a marker expressed selectively by colorectal tumors. The presence of GUCY2C in histologically negative lymph nodes could indicate the existence of occult metastases and better estimate recurrence risk. Here, 257 patients with pN0 colorectal cancer were enrolled prospectively at nine centers, providing 2,570 fresh lymph nodes greater than 5 mm in diameter for histopathology and quantification of GUCY2C mRNA by the reverse transcriptase-polymerase chain reaction (qRT-PCR). Patients were followed for a median of 45 months (range: 1-75) to estimate time to recurrence and disease-free survival. Thirty-two (12.5%) patients had lymph nodes negative by GUCY2C qRT-PCR [pN0(mol-)], and all but two remained free of disease during follow-up (recurrence rate 6.3% [95% Confidence Interval (CI), 0.8-20.8%]). Conversely, 225 (87.5%) patients had lymph nodes positive by GUCY2C qRT-PCR [pN0(mol+)], and 47 (20.9% [CI, 15.8-26.8%]) developed recurrent disease (p=0.006). Multivariate analyses revealed that GUCY2C expression in lymph nodes was the most powerful independent prognostic marker. Patients who were pN0(mol+) exhibited an increased hazard of earlier time to recurrence (adjusted hazard ratio 4.42 [CI, 1.05-18.53]; p=0.042) and disease-related events associated with reduced disease-free survival (adjusted hazard ratio 3.10 [CI, 1.09-8.82]; p=0.034). Thus, the positivity of histologically negative lymph nodes by GUCY2C qRT-PCR is independently associated with time to recurrence and disease-free survival in patients with pN0 colorectal cancer. GUCY2C may serve as an indicator of occult lymph node metastases, identifying pN0 patients at high risk for disease recurrence who might benefit from adjuvant chemotherapy.

Keywords: lymph nodes staging, prognostic and predictive markers, quantitative reverse transcriptase-polymerase chain reaction

497 Airway Epithelial Gene Expression in the Diagnostic Evaluation of Smokers With Suspect Lung Cancer

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Lung cancer is the leading cause of cancer death in the U.S. and world, with a five-year mortality of 80-85% due in part to a failure in early detection. Patients are referred to evaluation for lung cancer often due to concerning radiographic findings; with examination of materials collected via flexible bronchoscopy commonly used as an initial test to definitively diagnose cancer or other etiologies such as infection. The low diagnostic sensitivity of bronchoscopy (ranging from 30 - 70%) presents a clinical dilemma for the further evaluation of bronchoscopy-negative patients: either leading to the use of more definitive by invasive procedures that risk adverse complications, or repeat imaging studies that risk disease progression.

We have found that gene expression in cytologically normal epithelial cells collected from the mainstem bronchus at the time of diagnostic bronchoscopy differs between smokers with and without lung cancer, and that these differences can be used as a sensitive and specific biomarker of early stage disease to guide further diagnostic evaluation of bronchoscopy-negative patients [1,2]. We have further shown that a biomarker that incorporates the expression levels of 80 genes contains information about the likelihood of lung cancer that is independent of other clinical risk factors and physician assessment of lung-cancer risk and that a clinico-genomic model incorporating both clinical risk factors and gene expression has the best diagnostic performance [2].

In preparation for a clinical trial to further test the potential utility of gene expression in the diagnostic evaluation of suspect lung cancer, current work is focused on determining the relative performance of gene-expression biomarkers that incorporate the expression levels of fewer genes, and the possibility that lung-cancer-specific gene-expression heterogeneity also occurs in nasal epithelium. Additionally, we have begun to explore how a patient's airway gene-expression reflects the activation state of oncogenic pathways, potentially allowing for personalized approaches to chemoprophylaxis and therapy.

References: [1] Spira, et al. Nature Medicine. 2007. 13:361-6. [2] Beane, et al. Cancer Prevention Research. 2008. In Press.

Keywords: lung cancer, biomarker, gene expression

498 Gene Promoter Methylation in Lung Cancer: Identification of Genetic Determinants and Use in Disease Monitoring

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Gene promoter hypermethylation is a major mechanism in the etiology of lung cancer with more than 100 genes epigenetically silenced. The fact that gene methylation can be detected in sputum as first shown by our group and that methylation is reversible by pharmacological intervention has led us to initiate translational studies to assess whether methylation in sputum can be used as a biomarker for early lung cancer detection and predicting response to chemopreventive agents and to identify clinical covariates and genetic determinants that influence the propensity for methylation in the aerodigestive tract to better define mechanisms underlying the etiology of this cancer. The long-term goal of these studies is to integrate genetic susceptibility factors with epigenetic biomarkers and clinical risk factors for early detection of lung cancer. Our recent studies have identified double-strand break repair capacity (DRC) and specific genes within this pathway as determinants for gene methylation in sputum, which is in turn, associated with elevated risk for lung cancer. In addition, specific haplotypes of two of the major cytosine DNA methyltransferases (DNMTs), DNMT1 and 3B, were associated with a combined 50% elevation in mean level of chromosome breaks induced in lymphocytes from smokers by a tobacco carcinogen, supporting a major role for these genes in protecting the genome from DNA damage. Chronic obstructive pulmonary disease (COPD), a major risk factor for lung cancer, was also significantly associated with both increased prevalence for gene specific methylation and methylation index in the Lovelace Smokers Cohort. We have been conducting correlative laboratory studies to the "Phase III Chemoprevention Trial of Selenium Supplementation in Persons with Resected Stage I Non-Small Cell Lung Cancer" being conducted through the Cooperative Trial Network and led by the East Coast Oncology Group. This trial is testing the hypothesis that 200 μg of L-selenomethionine given as selenized yeast for 48 months can decrease the rate of second primary tumors in patients (n=1960) who have undergone curative surgery for stage Ia or Ib non-small cell lung cancer (NSCLC). For the laboratory studies, sputum and blood (plasma and mononuclear cells) are collected at entry onto the trial, and at 6, 12, 24, and 48 months during participation. Methylation of six genes (p16, MGMT, RASSF1A, GATA 5, PAX5 α, and PAX5 β) is currently being assessed in DNA isolated from sputum and plasma. Preliminary data were analyzed for methylation status at baseline for the initial 283 persons participating in the correlative laboratory studies who provided both a sputum and plasma sample. Median follow-up was 2 years (range, 0–5 years). Thirty-six events (local or extrapulmonary cancer or death) were seen in this population associated with 29 deaths. A Kaplan Meier plot depicting methylation of any gene (yes or no) revealed a significant association between methylation and event-free survival using the log-rank test ($p = 0.05$). These findings support our hypothesis that methylation in sputum could predict for cancer recurrence and even mortality. (Supported by U01 CA 097356 and R01 CA 095568.)

Keywords: methylation, lung cancer, biomarkers

499 RRM1/PTEN Axis in Response and Survival of Non-Small Cell Lung Cancer

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Advances in the management of patients with non-small-cell lung cancer (NSCLC) have been slow, and the 5-year survival rate is approximately 15%. Disease response to systemic chemotherapy, a key therapeutic modality for most patients, is 25-35%. We had identified the regulatory subunit of ribonucleotide reductase (RRM1) as a gene with potential impact on lung cancer pathogenesis and chemotherapeutic efficacy (Oncogene 22:2135, 2003; J Clin Oncol 22:1878, 2004). In a transgenic mouse model, we found that animals with RRM1 overexpression had reduced carcinogen-induced lung tumor formation and increased DNA damage repair capacity (Cancer Res 66:6497, 2006). A prospective clinical trial was conducted with the goal to study the association between *RRM1* gene expression and response to first-line gemcitabine/carboplatin therapy. In this trial, and related *in vitro* studies, *RRM1* was identified as the dominant predictive marker of treatment efficacy (J Clin Oncol 24:4731, 2006). *ERCC1* was confirmed as a second molecule associated with treatment response. We recently confirmed these results using routine tumor specimens prospectively collected on a large randomized community-based treatment trial (submitted). In another investigation, the prognostic impact and association between RRM1 and ERCC1 protein expression and survival of patients with surgically resected NSCLC was investigated. To accomplish this, reagents and technology were established and adopted. We found that high levels of RRM1, and in particular in association with high levels of ERCC1, are prognostic of long survival (N Engl J Med 356:800, 2007). In summary, we identified that high levels of RRM1 expression are prognostic of long survival but also predictive of poor response to gemcitabine and gemcitabine/platinum therapy. A phase II cooperative group trial is currently using RRM1 and ERCC1 expression levels for therapeutic decisions on adjuvant treatment in patients with stage I NSCLC. In addition, a randomized phase III trial in patients with advanced NSCLC is ongoing that builds on our prior experience using a tailored treatment approach (J Clin Oncol 25:2741, 2007).

Keywords: non-small cell lung cancer; RRM1; ERCC1

500 Strategic Partnering to Evaluate Cancer Signatures in Lung Cancer

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The goal of this program is to test candidate molecular signatures with potential clinical utility for the diagnosis, prognosis, or prediction of benefit from therapy in human lung cancer.

We have been developing and testing candidate serum biomarkers for the early detection and diagnosis of lung cancer patients using both comprehensive proteomic and candidate biomarker approaches. To date we have tested a 7-feature serum MALDI signature and have achieved an accuracy of 76% in test sets of stage I patients, and are currently testing signatures derived from shotgun proteomics. Initial analysis of a 40 marker serum cytokine panel has resulted in a 97% sensitivity and 77% specificity.

Biomarkers for the prediction of response or toxicity are being tested based on proteomic analysis of serum or tumor tissue, expression microarray analysis of tumors, and SNP analysis for inherited polymorphisms. One of our serum proteomics predictors for benefit from tyrosine kinase inhibitors has now been commercialized and is undergoing phase II and III testing within the SPECS consortium and in US and European cooperative group studies. A candidate proteomic signature from resected tumors predicting benefit from adjuvant chemotherapy is being tested. We have also completed analysis of 384 SNPs in DNA repair and cell cycle control genes in 1500 DNA samples from cancer patients treated with chemotherapy to test correlations with response and toxicity, and analysis of BRCA1 and nicotine receptor polymorphisms is underway.

Molecular diagnostic tests that can refine our diagnostic ability and aid in the selection of therapy will be crucial to rational application of targeted therapies and making progress toward improving the quality of life and survival of patients with cancer.

Keywords: optical imaging, biomarkers, chemoprevention

501 Tracing Clonal Lung Tumor Evolution Through Mitochondrial Footprints

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Lung cancer is a major health problem in the United States and interventions based on novel molecular detection strategies are gaining enthusiasm. In addition to nuclear genetic alterations, mitochondrial DNA (mtDNA) alterations have now been detected in a wide variety of tumors including lung cancer and have been suggested as a potential sensitive tool for cancer detection. In an attempt to scrutinize mtDNA alterations and their evolution, we examined the pattern of mtDNA mutation and mtDNA content index in 25 bronchoscopically abnormal airway mucosal biopsies and matched tumors from five resected lung cancer patients. The airway mucosal biopsies were histopathologically diagnosed as normal but exhibited multiple clonal mtDNA mutations which were detectable in the corresponding tumors. One of the patients was operated on twice for the removal of tumor from right upper and left lower lobe respectively within a span of two years. Both the tumors exhibited twenty identical mtDNA mutations and odds of this occurrence by chance were vanishingly low (6.9 x 1010: 1). The mtDNA content index also increased significantly ($P<0.05$) in the normal appearing mucosa and the corresponding tumors in all the patients. Our results trace the evolution of mtDNA alterations and their patterns through large patches of normal appearing mucosa shedding light on the strengths and weaknesses of mtDNA alterations as a possible tool for lung cancer detection and monitoring.

Keywords: lung cancer, mitochondria, molecular detection

502 Biomarkers Of Premalignancy And Detection of Early Lung Cancer

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Lung cancer is currently thought to result from a series of cellular and molecular changes in airway precursor cells that eventuate in invasive tumor. Characterization of these changes depends on the ability to access their site of origin in the lower airways and on application of cellular and molecular tests that may predict progression to invasive carcinoma. The Colorado EDRN Biomarker Development Laboratory has used a variety of tools to map the lower airway epithelium, characterize its histomorphology and test chromosomal and molecular lesions that may predict the future development of invasive lung carcinoma or prevalent invasive carcinoma. Assays for both blood and sputum have been tested, focusing mainly on central airway lesions.

In a series of 2,521 heavy smokers with obstructive airway disease who were observed over 9869 person years, we identified 174 incident carcinomas. Within this group, persons with sputum atypia of moderate grade or higher had an adjusted hazard ratio for developing lung cancer of 2.37. This figure may be considered a lower threshold in a search for biomarkers that could potentially predict future invasive lung carcinoma.

Individuals with moderate atypia have been offered white light and/or florescence bronchoscopy to better track changes in airway epithelium over time in an effort to identify molecular and cellular changes that may progress to lung cancer. To assist with this endeavor, the Colorado laboratory has worked with JPL/NASA to develop a web-based bronchial map that integrates imaging and other data from multiple platforms and provides a time/space framework for determining the fate of specific cell populations in high risk smokers. In preliminary studies we have been able to track lateral spread of squamous carcinoma *in situ* from specific foci in one lung through the airways to multiple foci in the contralateral lung over a period of two years. These data highlight the need to develop methods to treat “premalignant” lesions before they acquire the ability to spread and metastasize.

Molecular technologies that have been applied to airway epithelium have included tumor suppressor gene methylation and aneuploidy (FISH). Detection of sputum DNA methylation in 3 or more of 8 genes results in a hazard ratio of 4.5 at a time point 18 mos. before lung cancer diagnosis. Aneuploid detected in sputum cells using a multicolor probe set (LAVysion, Abbott Laboratories) at the same time point results in a hazard ration of 36. Additional methylation sites and FISH probes are currently being tested to improve the sensitivity and specificity of these assays. It appears that molecular assays can improve the predictive power of morphology alone but will probably benefit from further optimization of gene combinations and probe sets.

Finally, circulating biomarker assays are being developed to detect tumor cells in patients with existing carcinoma. The Colorado laboratory has concentrated for this effort on markers that are amplifiable and are strongly expressed in tumors but are absent in normal tissues and blood. Markers emerging from expression microarray analyses have included Cancer Testis genes (MAGE A, NY-ESO, TEX15 and XAGE1) and elongation factor EEF1A2 . This combination of markers distinguishes tumor homogenates from normal lung tissue with an AUC of 0.980. Whether this marker set will survive validation in sputum and peripheral blood and emerge as a significant early detection test is an open question at the moment. It seems likely that the marker set may have some value as a staging device but this use too will require additional validation.

Keywords: cytology, methylation, aneuploidy

503 Co-Amplification of MYC and EIF3H Significantly Improve Response and Survival in Non-Small Cell Lung Cancer (NSCLC) Treated With Gefitinib

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Background: MYC (8q24.21) and EIF3H (eukaryotic translation initiation factor 3, subunit H) (8q23.3) have been reported as amplified in numerous carcinomas. MYC is mostly a negative prognostic factor and MYC amplification seems to increase sensitivity to trastuzumab (Herceptin™), a monoclonal antibody against HER2, a member of the EGFR family. No data are available on EIF3H in lung cancer. We herein investigated the genomic status of MYC and EIF3H in NSCLC and their association with sensitivity to EGFR tyrosine kinase inhibitors.

Methods: A 3-color FISH probe set was used to investigate 54 metastatic NSCLC patients (N=54) treated with gefitinib. DNA sequences from MYC were labelled in SpectrumOrange, from EIF3H with SpectrumGreen and from CEP8 with SpectrumAqua.

Results: Amplification of EIF3H (ratio EIF3H/CEP8 > 2), was observed in 10 cases (18.5%), and MYC was co-amplified in all. MYC amplification without co-amplification of EIF3H was observed in 2 cases (3.7%). Response to gefitinib therapy was higher in MYC amplified than in non-amplified patients (25% versus 14%, p=0.4) and in EIF3H amplified versus non amplified (30% versus 14%, p=0.3). In order to investigate whether this trend for higher response was due to chance or reflected a significant biological difference, a Receiver Operating Characteristic (ROC) analysis was conducted to identify the cut-off for MYC and EIF3H copy number that best discriminated sensitive and resistant patient populations. MYC FISH positive patients (mean ≥2.79) had significantly higher response rate (RR: 31% versus 0%, p=0.003), significantly longer time to progression (TTP: 4.4 versus 2.6 months, p=0.01) and survival (OS: 13.8 versus 6.4 months, p=0.02) than MYC FISH negative patients (mean <2.79). EIF3H FISH positive patients (mean ≥2.75) had significantly higher RR (32% versus 0%, p=0.002), significantly longer TTP (4.4 versus 2.7 months, p=0.01) and OS (17.8 versus 6.4 months, p=0.01) than EIF3H FISH negative patients (mean <2.75).

Conclusions: MYC and EIF3H are frequently co-amplified in NSCLC and high copy numbers of these genes increase sensitivity to gefitinib therapy. Prospective validation of these findings is warranted.

Keywords: NSCLC, MYC, FISH

504 Circulating 25-Hydroxyvitamin D, VDR Polymorphisms, and Survival in Advanced Non-Small Cell Lung Cancer

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Vitamin D is a steroid hormone that has been shown in both *in vitro* and *in vivo* experiments to block cell growth. The hormonal activity of vitamin D is mediated by vitamin D receptor (VDR), which is a steroid hormone receptor. Several potentially functional polymorphisms have been identified in the human *VDR* gene. The *A* allele of the *Cdx-2 G>A* polymorphism (rs11568820) which is located in the *VDR* gene promoter region has been shown to have higher transcriptional activity. The *T* allele of the *FokI C>T* polymorphism (rs10735810), which is located in the translational initiation site of *VDR*, appears to be less efficient in exerting $1,25(OH)_2D$ effects as compared to the *C* allele. The *T* allele of the *BsmI C>T* polymorphism (rs1544410) has been associated with increased VDR mRNA expression and increased serum levels of $1,25(OH)_2D$.

A number of epidemiologic studies have suggested that vitamin D, or putative surrogates for vitamin D status such as geographic latitude or season, are associated with incidence and mortality of a variety of cancers. We investigated whether vitamin D levels and *VDR* polymorphisms were associated with survival outcomes in non-small cell lung cancer (NSCLC).

We first showed that season of surgery and dietary vitamin D intake were significantly associated with survival in Stage I-II surgically resected NSCLC. In addition, levels of circulating vitamin D correlated with survival in these patients. *VDR* polymorphisms also appeared to be associated with survival in early stage NSCLC, particularly among the squamous subset. Specifically, among patients with squamous cell carcinoma, carrying the *A* allele of the *Cdx-2 G>A* polymorphism, as well as increasing numbers of "protective" alleles in the *Cdx-2, FokI,* and *BsmI* polymorphisms, were associated with better survival. The *G-T-C* (*Cdx-2-FokI-BsmI*) haplotype, hypothesized to be associated with the lowest VDR expression or function, was also associated with worse survival in the squamous cell group.

We further investigated the association of vitamin D levels and *VDR* polymorphisms with survival among advanced stage NSCLC patients. We analyzed patients with incident cases of Stage III or IV NSCLC who were enrolled in an ongoing molecular epidemiology study at MGH and HSPH between December 1992 and July 2004. There were 294 patients and 233 deaths, with median follow-up of 42 months. We found no difference in survival by circulating vitamin D level. However, the *C/C* genotype of the *FokI* polymorphism was associated with improved survival: median survival for *C/C* 21.4 months, *C/T* 12.1 months, *T/T* 15.6 months ($p = 0.005$ by log rank). There were no significant effects on survival by the *Cdx-2* or *BsMI* polymorphism. However, having increasing numbers of protective alleles was associated with improved survival (AHR for $\geq$2 versus 0-1 protective alleles 0.57 (95% CI 0.41-0.79); p=0.0008). On haplotype analysis, the *G-T-C (Cdx-2-FokI-BsmI)* haplotype was associated with worse survival compared with the most common haplotype of *G-C-T*; AHR 1.61 (95% CI 1.21-2.14), p=0.001.

In summary, we found significant associations with *VDR* polymorphisms and survival in advanced NSCLC. As clinical trials with vitamin D in various cancers are undertaken, investigation of vitamin D levels and *VDR* polymorphisms will be helpful to confirm these findings.

Keywords: lung cancer, vitamin D, VDR polymorphisms

505 Shotgun Proteomic Analysis of Non-Small Cell Lung Cancer Tissue

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In-depth analysis of the cancer proteome should yield an unprecedented level of insight into dysregulation of cancer pathways as well as clues for the identification of diagnostic and therapeutic biomarkers. Previous technologies have allowed the analysis of only several hundreds of proteins, which is insufficient to reflect the complexity of the cancer proteome. We applied a novel shotgun proteomics approach combining peptide isoelectric focusing (IEF) and reversed phase liquid chromatography-tandem mass spectrometry (LC-MS-MS) to identify proteins differentially expressed between pools of 20 samples each from 2 lung cancer sub-types, adenocarcinoma (ADC) and squamous cell carcinoma (SCC) and normal lung from non-cancer controls.

With conservative criteria for protein identification from peptide sequences, we have confidently identified 3688 protein groups (6651 IPI accessions) from the 3 different tissue pools with quantitative information. Gene Ontology annotations indicated that 2487 intracellular proteins, 925 nuclear proteins and 514 plasma membrane or extracellular space proteins were identified. We observed 93% (tumor pool), 80% (normal pool) of all identified protein in at least 2 of 4 replicate analyses of each pool, indicating the high reproducibility of the analyses. The number of protein groups expressed uniquely in the ADC, SCC and normal tissue pools were 17, 117 and 21, respectively. Using spectral count data, we identified 232 differentially expressed proteins between the normal and tumor pools, and selected a subset of these known to be cell-surface or secreted proteins.

We have also developed a high-throughput, label-free mass spectrometric method for quantitation of up to 60 of these candidates simultaneously in complex biological samples to screen these proteins as candidate molecules for the early detection of lung cancer. In addition, to better understand the dysregulated pathways active in these cancers, we have identified the subset of these proteins with the greatest fold change between the tumor types and normal, and analyzed these using pathway analysis tools. Approximately 90% of this number of identifications can be obtained using formalin-fixed paraffin-embedded material, and clinical samples are being analyzed with this approach as well. Novel molecular alterations in lung cancer have already been identified using this approach.

Keywords: lung cancer, biomarkers, proteomics

506 Evaluation of Lung Cancer Chemopreventive Agents In Phase II Clinical Trials

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Serial bronchial biopsies are currently used to sample preneoplastic lesions to evaluate the effect of chemopreventive agents in Phase II clinical trials. The biopsy procedure itself can potentially introduce artifacts by mechanically removing these lesions. It is therefore important to develop non-biopsy methods that can determine the presence and progression/regression of preneoplastic lesions in the bronchial epithelium. Optical coherence tomography (OCT) is an optical imaging method that can offer microscopic resolution for visualizing cellular and extra-cellular structures at and below the bronchial surface. Bronchoalveolar lavage (BAL), performed in the same bronchoscopic procedure also allows measurement of biomarkers associated with regression or progression of preneoplastic lesions. OCT images and the corresponding bronchial biopsies were obtained from current and former smokers during autofluorescence bronchoscopy as part of Phase II trials. The results showed that invasive cancer can be distinguished from CIS and that dysplasia can be distinguished from metaplasia, hyperplasia or normal. Using quantitative measurement, a progressive increase in the epithelial thickness was found to parallel the severity of the histopathology grade. The nuclei of the cells became more discernible in lesions that are moderate dysplasia or worse. CC10 and surfactant protein D levels in the BAL fluid were found to be significantly correlated with regression/progression of bronchial dysplasia. Our study suggests that OCT is a promising non-biopsy tool for in-vivo imaging of pre-neoplastic bronchial lesions to study their natural history and the effect of chemopreventive intervention. Measurement of constitutents in BAL fluid can be informative regarding the effects of chemopreventive agents.

Keywords: optical imaging, biomarkers, chemoprevention

507 Molecular Signatures Predicting Response to Therapy in Lung Cancer

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We are working to identify mRNA, protein, miRNA, and DNA copy number and methylation profiles ("signatures") from individual lung cancers that predict to which drugs or radiation a patient's tumor is most likely to respond. We have develop drug response phenotypes (probability of being sensitive or resistance) for ~20 standard, targeted, and new chemotherapy agents and combinations for over 50 non-small cell lung cancer lines. We have profiled these same lines for genome wide mRNA profiles (with Illumina v2 arrays) and for over 50 proteins using reverse phase protein arrays (RPPAs). From this information we have developed mRNA and protein signatures predicting how lung cancer lines will respond or be resistant to these therapies in vitro with accuracies >70%. We have shown that the *in vitro* drug response phenotypes are also seen in orthotopic lung cancer xenografts *in vivo*. As part of this we have developed bioluminescence imaging (BLI) to follow the growth, response, and metastatic behavior of these lung cancer orthotopic xenografts. We are using this system to study over 45 NSCLC xenografts made directly from patient samples in a test of these signatures in "mouse preclinical" trials. mRNA profiling of over 40 of these xenografts has shown that the mRNA profiles are similarly predictive for drug response (R .0.9) as those from the lung cancer lines. As part of a joint NCI SPORE, NCI SPECS, and DOD PROSPECT and BATTLE effort we have identified a large number of clinically annotated frozen tumors with drug response information to test and validate these signatures including 131 that represent lung cancer resection followed by adjuvant treatment, and 109 specimens treated at the time of recurrence. These specimens are currently being profiled to formally test the clinical relevance of the tumor cell line and xenograft generated signatures. Finally, in collaboration with Vanderbilt and Moffitt Cancer Centers we have tested proteomics profiles generated by Dr. David Carbone from our NSCLC cell line panel to predict survival of NSCLC patients treated with adjuvant chemotherapy (platin + taxane) and find that patients whose tumors are predicted to be "sensitive" have significant better survival than those whose tumors are predicted to be "resistant." Thus, we are on the brink of developing mRNA and protein signatures predictive of the response of individual lung cancers to standard and targeted chemotherapy and also a new preclinical model system (orthotopic lung xenografts) to test new therapies *in vivo* in preclinical trials.

Keywords: lung cancer, predictive signatures, chemotherapy

508 Biomarkers for Lung Cancer Metastasis

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Lung cancer remains a significant public health issue and accounts for 28% of all cancer deaths in the United States. Therapeutic options and prognosis for non-small cell lung cancer (NSCLC) are primarily based on stage at presentation. However, staging is frequently inaccurate as approximately half of patients diagnosed with stage I disease actually have metastases at presentation and will die within 5 years. Identification of specific biomarkers that could ascertain which patients at presentation have metastasis would be an invaluable asset in patient management. In addition, the discovery of proteins involved in the development of metastatic disease has the potential to reveal therapeutic targets. Although we initially explored protein profiles for this purpose, our most recent efforts in the search for biomarkers of lung cancer metastasis have focused on regulatory T-cells as predictors of survival and on autoantibodies. In preliminary experiments, we have identified several novel autoantibodies that are associated with non-metastatic disease. The target proteins of two of these autoantibodies are known to be associated with a more aggressive malignant phenotype, suggesting that the autoantibodies may play a protective role against metastasis.

On the basis of these preliminary results, we propose to develop a microarray screening test for the diagnosis of metastasis based on the detection of a larger set of phenotype-specific autoantibodies. This project could have tremendous diagnostic and therapeutic significance in the management of patients with NSCLC. While this translational project will focus on an essential diagnostic issue in lung cancer, this model can be applied to many other issues in oncology.

Reference: Petersen RP, Campa MJ, Sperlazza J, Conlon D, Joshi M, Harpole DH, Patz EF. Tumor infiltrating $FOXP3^{+}$ Regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. Cancer 2006;107:2866-2972.

Keywords: lung cancer, biomarkers, autoantibodies

509 DNA Repair Enzymes Activity Biomarkers for Risk Assessment and Early Detection of Lung Cancer

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DNA repair has a key role in preventing mutations, and provides a major defense against cancer in humans as indicated by the high cancer predisposition of individuals with hereditary germ-line mutations in DNA repair genes. Several studies have shown that reduced DNA repair plays a similarly important role in sporadic cancers. However, the development of reliable, functional, and specific DNA repair biomarkers, which are suitable for epidemiological studies, is expected to facilitate progress in risk assessment and early detection of sporadic cancer.

Our goal is to develop a series of DNA repair biomarkers and apply them to cancer prevention in general, and lung cancer prevention in particular. Our approach is based on functional DNA repair assays, and specifically enzymatic DNA repair activities. We have previously developed an enzymatic activity assay for the repair of the oxidative DNA lesion 8-oxoguanine in extracts from human peripheral blood mononuclear cells (PBMC) (Paz-Elizur *et al.*, 2007). Using this assay evidence was obtained to indicate that reduced activity of the enzyme OGG (8-oxoguanine DNA glycosylase), which removes 8-oxoguanine from DNA, is a risk factor in lung (Paz-Elizur *et al.*, 2003) and head and neck cancer (Paz-Elizur *et al.*, 2006).

Under the EDRN NCI program we are developing additional blood tests for enzymes involved in the repair of oxidative DNA damage. These include the AP endonuclease APE1, which is common to most base excision repair sub-pathways, and MPG, a DNA glycosylase that removes etheno-adenine from DNA. Since MPG also removes hypoxanthine from DNA, we are developing in parallel MPG assays for both etheno-adenine and hypoxanthine, to examine whether there might be differential expression of its repair specificity in cancer risk. Emphasis is given to the development of reproducible, and quantitative assays. The role of inter-individual variations of these DNA repair activities in lung cancer risk will be examined in a pilot case-control study with 100 lung cancer patients and 100 matched control subjects. These DNA repair biomarkers are expected to be useful for a variety of cancers in addition to lung cancer.

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Keywords: DNA repair biomarkers, lung cancer risk, early lung cancer detection

510 Enhanced CD8 CTL Cross Priming as Vaccine Principle

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Gp96 is the one of the major protein and peptide chaperones of the endoplasmatic reticulum. It helps folding of membrane associated and secreted proteins and transports peptides on their way to MHC class I presentation. Srivastava was the first to show that gp96 isolated from tumor cells and injected into syngeneic mice was able to induce a tumor specific immune response that was able to protect mice from a subsequent challenge with the same tumor but not against other tumors. Gp96 is taken up by dendritic cells and macrophages via its endocytic receptor CD91, resulting in the activation of DC, independent of CD4 cells and CD40-L/CD40 interaction. Uptake of gp96 and its chaperoned peptides results in cross presentation of the peptides by MHC I of the DC and priming of antigen specific, cognate CD8 T cells with over one million fold higher efficiency than intact protein.

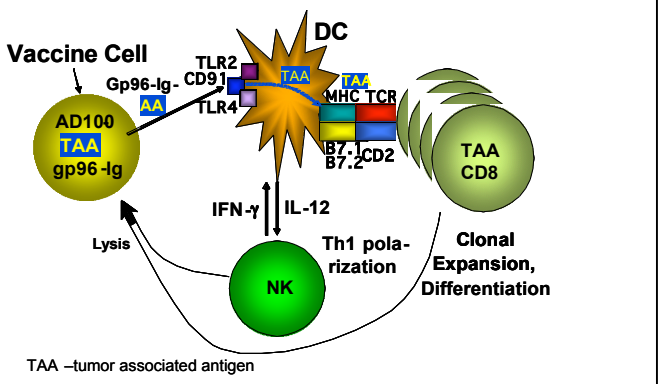

Figure 1: Putative activation of CD8 CTL by gp96-Ig-peptide

To make gp96 suitable in a vaccine system with allogeneic cells we genetically modified the protein by deleting its endoplasmatic reticulum retention signal and replacing it with the Fc portion of IgG1. Gp96-Ig transfected tumor cells secrete gp96-Ig along with its chaperoned peptides. Injection of gp96-Ig transfected tumor cells into syngeneic mice results in tumor rejection associated with the clonal expansion of cognate CD8-CTL to a frequency of 15-40% of all CD8 cells (4). Clonal CD8 CTL expansion is enhanced in CD4 deficient mice while CD40-L deficient mice are similar to w.t. mice. The combined knock out of B7.1 and B7.2 completely abrogates CD8 expansion while the loss of either single gene reduces expansion to about 50% of w.t. NKT cells are not required but NK cells enhance CD8 CTL responses. Antigen cross presentation and cross priming of cognate CD8 cells is ~20 million fold enhanced when peptides are chaperoned by gp96 compared their presence as intact protein, as measured with ovalbumin TCR transgenic system. Importantly, gp96-Ig mediated antigen specific cross priming works efficiently in the absence of lymph nodes in LTα deficient mice.

Following the events after intraperitoneal injection of tumors secreting gp96-Ig, we found that gp96-secretion promotes recruitment of monocyte/macrophages, dendritic cells and NK cells. DC and NK cells become activated only when gp96 is secreted by the injected tumor cells leading to CD8 CTL expansion initially locally followed by systemic expansion. Lymphotoxin α k.o. mice have no peripheral lymph nodes including mesenteric lymph nodes and Peyer's patches, however they are able to support gp96-Ig mediated CD8 CTL expansion to a similar extent as wild type mice. Using the ovalbumin as surrogate antigen, alllogeneic tumor cells transfected with ovalbumin and gp96-Ig are able to mediate cognate CD8 CTL (OT-I) expansion to the same extent as syngeneic cells. Gp96-Ig deliverd by allogeneic tumor cells thus has many properties that make it suitable for vaccine purpose.

We have cotransfected the AD100 NSCLC line with HLA A1 and gp96-Ig. One million cells secrete 400ng gp96-Ig within 24h in tissue culture. Upon irradiation with 12,000 rad the cells continue to secrete gp96-Ig at declining rates for the next two weeks in culture. In the phase I study that has just opened we will enroll 24 patients with stage IIIB/IV NSCLC who have failed at least two lines of prior therapy. Early results will be presented and further vaccination strategies discussed.

Keywords: lung cancer, chaperones, CTL

511 Validation of Patterns of Protein Expression in Preinvasive Lung Lesions and Identification of Biomarker Signature of Lung Cancer Development

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A biomarker signature of lung cancer development is warranted for early detection. To this goal we analyzed proteomic profiles by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) from normal bronchial epithelium, preinvasive lung lesion and invasive lung tissues. We hypothesized that the airway epithelium modifies its protein expression profile early during tumor development and that we would find a pattern of expression specific to subgroups of preinvasive lesions predictive of developing into cancer. From previous signatures validated across 3 datasets, we selected a signature of 11candidate features (m/z values) serving as best predictor of preinvasive lesions closely associated with an invasive phenotype. This signature has a 75% to 95% prediction accuracy to distinguish normal epithelium and low grade preinvasive from high grade preinvasive and invasive lung lesions. The proteomic profiles of these tissues were distinct from each other within a disease continuum. We have identified 8 out of these 11 candidate m/z values. Over-expression of these proteins in lung tumor tissues was confirmed by immunohistochemistry. We localized by MALDI imaging technique candidate proteins across a unique tissue specimen that harbors all histological stages of progression from normal epithelium to preinvasive lesion and invasive lung cancer. The validation of the proteomic signature in an independent dataset and the identification of the candidates may provide rationale to advance this signature towards clinical utility for the early diagnosis of lung cancer. Funding: RO1 CA 102353.

Keywords: lung cancer, biomarkers, tissue microarray, preinvasive lesion

512 Reactivity to Tumor Associated Antigens in the Evaluation of Pulmonary Nodules

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One hundred thirty six high risk asbestos exposed smokers enrolled in the New York University (NYU) Lung Cancer Biomarker Center were evaluated with CT scans at the time of entry into the cohort. Based on these CT scans, the participants were classified into 4 groups: no nodules (n=35), solid nodules (n=55), and ground glass opacities (GGO, n=46). An additional group of 22 lung cancer cases were identified upon referral to a thoracic surgeon. A fifth group of 36 healthy non-smokers were enrolled at the Scripps General Clinical Research Center. Ten tumor associated antigens (TAA) enzyme immunoassays on sera were obtained at the time of the initial CT scan for all of these subjects. These potential biomarkers are: p53, c-myc, imp1, p62, imp3 (derivatives of IGF-II), cyclin A, cyclin B1, cyclin D1, cdk2, and survivin. The focus of this report is to examine the potential to distinguish lung cancer based on these 10 biomarkers in this group of smokers at high risk of lung cancer.

The four groups of high risk subjects were compared with respect to the distributions of age, sex, smoking history (pack-years), FEV1/FVC, and history of other diseases including emphysema, lupus, and occupational asbestos exposure. The 22 cancer patients were older (median age of 65 years) and 50% were males compared with other subjects (median ages of 56-59) and 49-43% males, and 41% had emphysema compared with 31% of the no nodules and solid nodules groups and 46% of the GGO group. The statistically significant differences in the distributions of each of the biomarkers were identified among all five groups (Kruskal Wallis ANOVA, $p \leq 0.008$). A series of logistic regression models based on the biomarker levels among the high risk asbestos exposed and lung cancer groups were examined to identify the combinations of biomarkers that best distinguished among the groups. Receiver Operating Characteristic (ROC) curves were used to identify the optimal cutoff for sensitivity and specificity of the logistic scores and the resulting models were then applied to the healthy non smoking control group. For example, based on age, c-myc, Cyclin A, Cyclin B1, Cyclin D1, CDK2, and survivin, we obtained a sensitivity = 81% and specificity =97% for the classification of cancer vs (no nodules, solid nodules, or GGO) and correctly predicted 34/36 healthy controls as noncancer. When we compared cancer patients with (no nodules or solid nodules only), based on c-myc, Cyclin A, Cyclin D1, and cdk2 we correctly predict 100% of the control patients. These models are being refined and validated.

Keywords: autoantibodies, lung cancer, biomarker

513 Systemic Therapy With Tumor Suppressor FUS1-nanoparticles for Stage IV Lung Cancer

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The tumor suppressor gene FUS1 is frequently inactivated early in the development of lung cancer. We examined Fus1 immunohistochemical (IHC) expression in 301 lung cancers and loss and reduction of expression was detected in 100% of SCLCs (N = 22) and 82% of NSCLCs (N = 281), without significant differences between adenocarcinoma and squamous cell carcinoma histologies. In NSCLCs, loss of Fus1 IHC expression was associated with significantly worse overall survival. FUS1 mediates apoptosis in cancer cells but not normal cells through its interaction with Apaf1 and down regulates tyrosine kinases including EGFR and c-abl. We developed a DOTAP:cholesterol nanoparticle encapsulating an optimized FUS1 expression plasmid that showed selective uptake by fresh cancer cells compared to normal cells and mediated tumor regression in orthotopic xenograft mouse models. In this clinical trial a FUS1 expression plasmid in a DOTAP:cholesterol nanoparticle was injected intravenously in stage IV lung cancer patients who had received cisplatin combination chemotherapy and showed tumor progression at the time of entry into the study. Nanoparticle-DNA complexes were manufactured in GMP facilities to meet specifications of OD400, size, appearance, and transfection efficiency. Patients received doses ranging from 0.01-0.06 mg/kg at 3 week intervals for a maximum of 6 doses. Dexamethasone and diphenhydramine premedications were added and eliminated the only clinically significant toxicity of fever. To date 21 patients have been entered on the study at five different doses (0.1, 0.02, 0.03, 0.04, 0.06 mgDNA/kg). All patients could be evaluated for the primary endpoint of toxicity, and with pretreatment, there was no significant drug related toxicity. Fifteen patients received two or more doses and could be evaluated for response with 1 patient showing tumor regression and 3 patients achieving stable disease (3 – 11 months). A maximum tolerated dose (MTD) has not been reached. Pre and 24 hour post-treatment tumor biopsies were obtained from 3 patients by percutaneous computed tomographic guidance from a central tumor location. A quantitative real time reverse transcriptase PCR (RT-PCR) analysis using a plasmid FUS1 sequence-specific probe was performed on samples blinded to time of biopsy. A high level of plasmid FUS1 expression was detected in all 3 post-treatment samples but not in three pretreatment samples and negative controls by RT-PCR. An estimated 4 copies of the FUS1 gene per cell were detected in a specimen from a patient receiving 0.01mg DNA/kg. When the dose was increased to 0.02mg/kg the copy number per cell increased to 20 and 28 copies per cell in two patients. DOTAP:cholesterol FUS1 nanoparticles can be safely administered intravenously in patients with stage IV lung cancer. Gene expression is detected in tumors, and there is an indication of anti-tumor activity. We found that re-expression of wild-type FUS1 by *FUS1*-nanoparticle-mediated gene transfer into FUS1-deficient and gefitinib-resistant NSCLC cell lines, including K-ras mutants, sensitized their response to gefitinib treatment and synergistically induced apoptosis *in vitro* and in an orthotopic lung cancer mouse model. FUS1 nanoparticle treatment alone or with gefitinib inactivated EGFR and AKT, as shown by decreased phosphorylation levels of both proteins on Western blots, compared with either agent alone. Cleavage of caspase-3, caspase-9, and PARP was also significantly induced by the combination of FUS1 and gefitinib in HCC872GR and other gefitinib-resistant NSCLC cells. The combination of FUS1 and erlotinib induced similar levels of tumor cell growth inhibition, apoptosis induction, and inactivation of oncogenic PTKs as those observed in NSCLC cells treated by a combination of FUS1 and gefitinib. These studies provide the rationale for a planned clinical trial combining FUS1 nanoparticles and erlotinib in stage IV lung cancer patients.

Keywords: gene therapy, nanotechnology, lung cancer

514 A Proteomic Approach to Early Detection of Lung Cancer

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New measures are crucial for early detection of lung cancer. The University of Colorado Cancer Center (UCCC) developed recently together with Vanderbilt University and the company Biodesix (Steamboat Springs, Colorado) a proteomic classifier for prediction of outcome to EGFR TKIs in the treatment of advanced Non-Small Cell Lung Cancer (NSCLC) (Taguchi et al. JNCI 99 (11): 838-46.2007).

At UCCC a high-risk cohort of individuals (smoking history> 30 pack-years and FEV1< 75%) have been followed since 1992 and about 4.000 individuals have been followed with baseline and yearly sputum examinations and repeated bronchospies if sputum showed moderate atypia or worse or if there were other clinical indications (within the last years also including spiral CT). From the same cohort peripheral blood were stored. Together with Biodesix we are studying serum proteomics in order to develop a classifier, which can be used for early detection of lung cancer. Altogether, 293 individuals are included in the preliminary case-control study (test set and validation set): The control group includes : never smokers (N=60), smokers with COPD (N=60), smokers without COPD (N=60) and the case group includes: patients with adenocarcinoma (N=61) and with squamous cell carcinoma (N=52). The specimens are identified for the algorithm development and the test set, but blinded for the validation set. Encouraging data are already established and results from the validation set will be ready during the fall of 2008.

Keywords: lung cancer, biomarkers, tissue microarray

515 The Zinc-Finger E-Box-Binding Transcriptional Repressor Snail Links Inflammation, Angiogenesis, and EMT in Human NSCLC

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The pulmonary environment at risk for carcinogenesis contains inflammatory mediators capable of potently promoting epithelial-mesenchymal transition (EMT) through the induction of E-cadherin transcriptional repressors, including the zinc finger transcription factor family member Snail. Snail gene-modified human bronchial epithelial cells (HBEC) were utilized to define early events in lung carcinogenesis. Snail was up-regulated and E-cadherin was concomitantly down-regulated in HBEC Snail cells (HBEC-S) compared to HBEC vector control cells (HBEC-V). Compared to HBEC-V, HBEC-S cultures expressed significantly elevated concentrations of the angiogenic proteins CXCL5 and CXCL8. In a three-dimensional (3D) spheroid culture model, HBEC-V formed compact spheroidal colonies, whereas HBEC-S formed stellate cell aggregates with invasive processes. In a 3D organotypic culture model, HBEC-V formed well-organized epithelial cell layers atop a modified basement membrane. In contrast, HBEC-S epithelial cell layers were disorganized, discohesive, and exhibited invasive behavior. Immunohistochemical (IHC) staining of E-cadherin was intense in HBEC-V but nearly absent in HBEC-S. Staining with the basal markers, Cytokeratin 14 and p63, was appropriately restricted to the basal epithelial cell layer in HBEC-V cultures. In contrast, HBEC-S cultures were uniformly positive for the markers regardless of the location of the cells within the epithelium and underlying mucosa, suggesting a loss of polarity. These findings demonstrate that over-expression of Snail in normal human lung epithelium results in down-regulation of E-cadherin and induction of subsequent molecular and morphological changes characteristic of EMT. Microarray gene expression analysis indicates that Snail initiates a program of inflammation, invasion, and angiogenesis. To define the role of Snail in established lung cancer, we utilized Snail gene-modified NSCLC cell lines. Two human NSCLC cell lines, H441 and H292, were stably transduced with a Snail over-expressing retroviral vector (pLHCX). Severe combined immunodeficiency (SCID) mice were injected subcutaneously with vector control and Snail up-regulated cells. Consequent primary tumor burden was significantly greater in Snail up-regulated tumors compared to vector control tumors ($p<0.005$), but the enhanced tumor growth was reversed when mice were treated with antibody to CXCR2. By ELISA, three angiogenic proteins, CXCL5, CXCL8, and VEGF, were elevated in the homogenates of Snail tumors compared to vector tumors. Microarray gene expression analysis performed on in vitro samples revealed 1000 fold differences in gene expression between vector control and Snail up-regulated cells for genes known to influence tumor growth, invasion, and angiogenesis, including EGR-1, TSP-1, and SPARC. These findings suggest that Snail expression plays an important role in the pathogenesis of NSCLC by serving as a critical link between inflammation, angiogenesis, and EMT.

[These studies were supported by UCLA SPORE in Lung Cancer P50CA90388 and UT Southwestern SPORE in Lung Cancer P50CA75907. Keywords: lung cancer; Epithelial-mesenchymal transition; Snail]

Keywords: snail, non-small cell lung cancer, angiogenesis

516 Lung Cancer Diagnostic Test Validation in Transthoracic Fine Needle Aspirate Specimens

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Lung cancer is the most common cancer cause of death in this country. At the time of diagnosis, it usually is too advanced to be treated effectively with surgery. In these advanced cases, diagnosis is made by the least invasive, non-surgical methods available. This typically involves morphologic analysis of cells obtained by transthoracic fine needle aspirate (FNA) or bronchoscopic biopsy. Although it is the standard of care, cytomorphologic analysis of these small specimens is only approximately 80% sensitive for lung cancer, does not always allow morphologic sub-classification, and provides no information regarding expected survival outcome. Gene expression analysis is likely to improve diagnostic accuracy and provide information that will enable improved management. However the small cytology specimens obtained by FNA or bronchoscopy are not sufficient for most gene expression assay methods. In recent studies, we have developed methods for measuring expression of hundreds of genes in RNA from small cytology specimens, including improved techniques for preserving and extracting RNA combined with a quantitative RT-PCR method optimized for clinical diagnostic testing, standardized RT (StaRT)-PCR. Using these methods in preliminary tests, the E2FI x c-myc/p21 interactive gene expression index, after analysis of 138 samples, a cut-off of 15,000 for the [MYC X E2F1]/p21 malignancy index correctly classified 124/138 samples for an observed correct classification rate of 90% (95% CI 83.6% - 94.3%). For this cut-off, the Sensitivity was 86.2%, and Specificity was 92.5%. Based on these results, the [MYC X E2F1]/p21 test represents a significant improvement in accuracy of lung cancer diagnosis based on existing cytomorphologic criteria. It is likely that combining both tests will result in optimal accuracy. In spite of these promising results, it was clear from these studies that inadequate control for inter-sample variation in RNA quality reduced the accuracy of the [MYC X E2F1]/p21 test compared to what we might otherwise have achieved. In the next phase of study, we will a) validate promising new methods for controlling for inter-sample variation in RNA quality, and b) validate the [MYC X E2F1]/p21 test in another cohort of patients. We expect that the proposed project will validate our methods for obtaining detailed gene expression information from each individual tumor prior to treatment, whether from cytologic or histologic specimens. We expect that validation of these sample collection and assessment methods will enable development and validation of additional promising molecular diagnostic tests overall outcome and response to treatment in every case. We expect that diagnostic tests resulting from these studies will reduce cost and suffering related to lung cancer diagnosis and treatment and will lead to improved design of clinical trials.

Keywords: lung cancer, standardized RT-PCR, RNA quality control

517 Molecular Classification of Non-Small Cell Lung Cancer (NSCLC) for Prognosis and Response to Treatment Prediction

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Over the course of more than 10 years, we have established a large collection (~2,500) of surgically resected (stages I-IIIA) frozen and archival formalin-fixed and paraffin-embedded (FFPE) NSCLC tissue specimens, with a subset of ~1,000 cases having annotated clinical information, including a detailed smoking history and follow-up. Using these resources, we have aimed to develop an integrated histopathological and molecular classification of NSCLC to better prognosticate survival of patients and predict response of tumors to standard cytotoxic and targeted therapies. In addition, we have aimed to characterize molecular changes in NSCLC metastasis sites compared to primary tumors. To accomplish these aims, and as part of joint NCI-SPORE and DoD VITAL, IMPACT and PROSPECT grants, we have developed: a) a set of FFPE 330 NSCLCs in tissue microarrays (TMA) used for immunohistochemistry (IHC) analysis of over 120 markers (including among others, oncogenes, tumor suppressor genes, proliferation, DNA repair, stem cells and nuclear receptor markers), gene copy number by fluorescent *in situ h*ybridization (FISH)/quantitative PCR (qPCR) examination of *EGFR*, *HER2*, *TITF-1* and *NOTCH3*, and mutation analysis of *EGFR* and *KRAS*; b) a large set of DNA and RNA collection obtained from 750 frozen NSCLC with annotated clinical information, including neoadjuvant and adjuvant therapy regimens, to be used for profiling analysis at DNA, RNA and protein levels; and, c) a collection of FFPE NSCLC tissues containing primary tumors and metastasis to brain (N=60) and other sites (N=100). The tissues from these NSCLC have been extensively characterized for histopathology features, including tumor cell content and histological subtypes of adenocarcinoma histology. From our NSCLC TMA set, we have identified multiple markers and several pathways' activation that associate with patients' clinical and pathological characteristics, including recurrence-free and overall survivals, and tumor genetic properties, especially *EGFR* mutation and *TITF-1* amplification. By our ongoing bioinformatic analysis, we expect to develop an integrated clinical-pathological-molecular signature correlating with NSCLC patients' outcome. Our NSCLC DNA-RNA sets are currently under profiling analysis to test the clinical relevance of the molecular signature to predict response to adjuvant therapies in lung cancer. Finally, using our NSCLC metastasis we have identified important differences in the expression of markers comparing brain metastasis and primary tumors, especially for EGFR and epithelial-mesenchymal transition (EMT) abnormalities.

Keywords: lung cancer, biomarkers, tissue microarray

518 PSMA-VRP Vaccine for Prostate Cancer

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Prostate cancer is the most common cancer affecting men and accounts for ~30,000 deaths annually in the United States. The primary organ-confined cases can effectively be treated and cured by surgery and/or radiation therapy. Relapsed or more advanced disease can be controlled temporarily with androgen ablation. However, in virtually all patients, the tumor ultimately becomes hormone refractory. The only approved chemotherapy for hormone-refractory disease (docetaxel in combination with prednisone) provides a modest survival benefit. Therefore, there is an urgent need for novel, molecularly targeted therapies for advanced prostate cancer.

One of our approaches is to develop a vaccine based on prostate-specific membrane antigen (PSMA). PSMA is a type II transmembrane glycoprotein that is abundantly and preferentially expressed in prostate cancer cells, and its expression is highest in progressive and metastatic disease. Our vaccine, PSMA-VRP, is a propagation-defective vaccine replicon particle (VRP) vector system, derived from an attenuated strain of the alphavirus Venezuelan Equine Encephalitis virus. The genetic region encoding the structural proteins of this attenuated strain has been replaced with the full-length human PSMA gene. Since the structural protein genes have been replaced with the heterologous gene insert, the vaccine replicon particle is designed to be restricted to a single cycle of replication. Alphavirus replicon vectors offer a number of advantages for vaccine delivery and have elicited protective immunity to a variety of pathogens and malignancies in animal models.

In preclinical studies, PSMA-VRP directed high-level expression of PSMA that was presented in a native conformation on the surface of infected cells. In mouse immunogenicity studies, PSMA-VRP elicited robust, dose-dependent humoral and cellular immune responses to PSMA, and the responses were boosted following repeat immunizations.

These results support clinical testing of PSMA-VRP in men with advanced prostate cancer.

Keywords: prostate cancer, PSMA, cancer vaccine, alphavirus

519 Efficacy of Salvage Chemotherapy for Small Cell Lung Cancer is Enhanced With a p53-Targeting Tumor Vaccine

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We conducted a clinical trial to test the safety and efficacy of a dendritic cell vaccine using p53 as a tumor antigen in SCLC patients. The rationale for using p53 as a tumor antigen is that p53 is mutated in 90% of SCLC, and the mutation produces a non-functional but stable p53 protein that is markedly over expressed. We sought to exploit this differential expression in tumor cells as compared to normal cells. The vaccine, Ad.p53-DC, was well tolerated. The majority of patients (57.1%) were observed to have p53-specific CTL responses and two patients demonstrated a clinical response to vaccination. A high rate (61.9%) of objective clinical responses to chemotherapy, particularly paclitaxel, that immediately followed vaccination was observed and correlated positively with immune responses. We set out to test the hypothesis that the Ad.p53-DC/paclitaxel combination therapy enhances the killing of SCLC cells by increasing the sensitivity of SCLC to p53-specific CTL-induced apoptosis. To begin to address this hypothesis, we evaluated surface expression of death receptors (DRs) (Fas, TNF-R, and DR4/5) as well as the regulation of key apoptosis-associated molecules in SCLC cells treated with paclitaxel. Immunophenotyping demonstrated that paclitaxel treatment of SCLC cells induces the surface expression of DRs. This upregulation sensitizes SCLC cells to TRAIL mediated apoptosis and Western blot analyses of paclitaxel/TRAIL-treated SCLC cell lysates revealed the involvement of Bcl-xl. Studies to further characterize the pathway(s) involved are currently being performed.

Keywords: tumor vaccine, small cell lung cancer, chemotherapy

520 Targeting Novel Prostate Tumor Antigens for Cancer Immunotherapy

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Background: Failure of immunotherapy for prostate cancer (PCa) in clinical trials in the past is partly due to the lack of a causative oncogene antigen target for such trials, and to the inability to overcome the various immunological checkpoints that govern immune tolerance to tumor antigens. Recently identified gene fusion of TMPRSS2 with the ETS transcription factor ERG in PCa and subsequent evidence that ERG is a putative PCa oncogene suggests the possibility of inducing effective PCa immunotherapy via the induction of CTLs specific for ERG-derived peptides expressed by human class I MHC. Additionally, identification of the T lymphocyte membrane receptor Tim-1, and evidence that its stimulation enhances antigen-specific T cell responses and overcomes immune tolerance to antigen, which suggests that manipulating Tim-1 pathway might boost the immune response to tumor antigens and ultimately benefit immunotherapy for PCa.

Objective: We now hypothesize that targeting novel putative prostate TAA in combination with interference with immunosuppressive mechanisms by either manipulating Tim-1 pathway or androgen ablation would benefit prostate cancer immunotherapy. **Methods:** Identification and validation of novel putative TAA was performed using gene expression profiling and quantitative RT-PCR. ERG- and SIM2-derived, HLA-A*0201-restricted epitopes were predicted using prediction algorithms, tested in vitro for binding to HLA molecules, and tested for immonogenicity in HHD humanized HLA-A*0201 transgenic mice. Manipulation of Tim-1 is achieved with monoclonal antibodies, fusion proteins, or Tim-4-expressing dendritic cells. Immunization and immunotherapy protocols using peptides, in combination with manipulation of Tim-1 and androgen pathways were tested in transgenic mice that express the target TAA and exhibit human HLA-restricted antigen presentation. **Results:** Our in silico studies unraveled 57 novel prostate TAA, and identified putative HLA-A2.1 restricted epitopes of ERG and SIM2. We carried out in vitro studies that show binding of these peptide epitopes to human A2.1, and finally studies in which we demonstrated the ability to overcome TAA-specific tolerance to the prostate TAA's ERG and SIM2 following immunization by these peptides in vivo. Additionally, we demonstrated the ability of circumventing immune tolerance to a model TAA by targeting Tim-1 receptor on T cells in TRAMP mice, and by castration of PSA/A2.1 transgenic mice. **Conclusion:** Our findings, demonstrating the ability to overcome T cell tolerance against human HLA A2.1-restricted epitopes of the prostate cancer associated tumor antigens ERG and SIM2, implicate these antigen epitopes as potential substrates for clinical trials of prostate cancer immunotherapy.

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Keywords: immunotherapy, transcription factors, transgenic mice

521 Vaccination of Patients With Metastatic Renal Carcinoma With Dendritic Cell/Tumor Fusions Following Debulking Nephrectomy

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We have developed a cancer vaccine in which patient derived tumor cells are fused with autologous dendritic cells (DCs) such that a broad array of tumor antigens are presented in the context of DC mediated costimulation. While initial clinical studies demonstrated immunologic response to vaccination, efficacy was limited by tumor mediated immune suppression. In metastatic renal carcinoma (RCC), debulking nephrectomy has been associated with improved outcomes potentially due to the resulting enhancement of host immunity. We are conducting a phase I trial in which patients with metastatic RCC undergo vaccination with autologous DC/RCC fusions following debulking nephrectomy. An initial cohort underwent vaccination with DC/RCC fusions alone and, in the absence of significant toxicity, a subsequent cohort underwent vaccination in conjunction with GM-CSF. To date, 21 patients have been enrolled and 13 patients have undergone successful vaccine generation. RCC were isolated from nephrectomy specimens that underwent mechanical and chemical digestion and cryopreserved as single cell suspensions. DCs were generated from adherent mononuclear cells isolated from leukapheresis collections cultured for 5 days with GM-CSF and IL-4 and matured by exposure to TNFα for 48-72 hours. The mean yield of DCs was 181 x 10^6 with a mean viability of 82%. DC preparations expressed costimulatory and maturation markers. RCC were thawed and expression of the tumor associated markers, cytokeratin and MUC1 was confirmed. At time of fusion, mean RCC yield was 40 x 10^6 cells with a mean viability of 81%. Fusion cells were quantified by determining the percentage of cells that co-expressed unique DC and RCC antigens following coculture of RCC and DCs with polyethylene glycol. Mean fusion efficiency, viability, and vaccine dose was 29%, 79%, and 3.4 x 10^6 fusion cells, respectively. Similar to the DC preparations and in contrast to unfused RCC, DC/RCC fusions potently stimulate allogeneic T cell proliferation ex vivo (mean stimulation indexes of 106, 47, and 11 for the DC, fusion and RCC preparations, respectively). To date, 11 patients have completed vaccination, two additional patients are currently undergoing vaccinations. Adverse events potentially related to vaccination were largely restricted to injection site reactions. All patients in the initial cohort undergoing evaluation demonstrated vaccine induced anti-tumor immunity as defined by a minimum 2 fold increase in IFNγ expression by CD4 and/or CD8 T cells in response to ex vivo exposure to autologous tumor lysate. Mean circulating levels of regulatory T cells are being quantified at serial time points. Of 11 patients evaluable for clinical response, 3 patients demonstrated a partial response, and 3 have stable disease lasting 15, 9, and 3 months following final vaccination. In preliminary analysis, vaccination with DC/RCC fusions following nephrectomy was feasible, well tolerated, and associated with immunologic and clinical responses.

Keywords: dendritic cell/tumor fusion vaccine, tumor immunotherapy, renal cell carcinoma

522 Delivery of HPV DNA Vaccine via Electroporation Results in the Most Potent Anti-Tumor Responses Compared to Intramuscular Injection and Gene Gun Delivery

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DNA vaccines are a promising approach to antigen-specific cancer immunotherapy. Intracellular targeting of antigens via an immunostimulatory molecule such as calreticulin (CRT) can improve antigen presentation and cytotoxic CD8+ T cell production; however, the efficacy of immunotherapy is limited by the route of administration of the DNA vaccine. Electroporation and gene gun-mediated particle delivery are leading methods of DNA vaccine delivery that can generate protective and therapeutic levels of immune responses in experimental models. In this study, we perform direct comparisons of the ability of three methods of vaccination - intramuscular injection, electroporation, and gene gun-mediated particle delivery - to generate antigen specific cytotoxic CD8+ T cell responses as well as generate anti-tumor immune responses against an HPV-16 antigen expressing tumor cell line (TC-1) using the pNGVL4a-CRT/E7(detox) DNA vaccine in mice. As human clinical translation is of paramount concern in this study of a clinical grade vaccine, a weight adjusted dose of 2 μg of pNGVL4a-CRT/E7(detox) DNA vaccine was administered via all vaccination methods. Vaccination via electroporation generated the highest number of E7-specific cytotoxic CD8+ T cells, which correlated to improved outcomes in tumor treatment. Additionally, we demonstrate that electroporation results in significantly higher levels of circulating protein compared to gene gun or intramuscular vaccination, which likely enhances calreticulin's role as a local tumor anti-angiogenesis agent. Therefore we conclude that electroporation is a promising method for delivery of HPV DNA vaccines.

Keywords: DNA vaccine; calreticulin (CRT), human papillomavirus (HPV), electroporation

523 A Phase I/II Trial Testing Immunization With AFP + GM-CSF Plasmid Prime and AFP Adenoviral Vector Boost in Patients with Hepatocellular Carcinoma

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Our objective is to evaluate the safety, toxicity and immunological effects of adjuvant administration of priming with three intramuscular administrations of a plasmid expressing human AFP (phAFP) with a plasmid expressing human GM-CSF (phGM-CSF), followed by a single intramuscular boost with an AFP adenoviral vector (AdVhAFP) to patients with locoregionally pre-treated hepatocellular carcinoma (HCC). We will enroll HLA-A2.1 positive patients with AFP-expressing HCC who received locoregional therapy, from stage II to IVa, and vaccinate a total of 25 patients. Our primary endpoints are Dose Limiting Toxicity (DLT) and Phase II Recommended Dose (P2RD) and immunological response rate in PBMC as indicated by the ELISPOT assay. Our secondary endpoints include: Disease-Free Survival (DFS), immunological response rate by MHC tetramer assay and immunological response rate in lymph nodes by ELISPOT assay.

We will utilize state-of-the-art immunological assays to detect responses in peripheral blood. By immunizing with the entire 1.8 kb gene, we will stimulate a broad response encompassing CD8+ T cells specific for immunodominant and subdominant epitopes, as well as AFP-specific CD4+ T cells. We will analyze these cells for frequency, cytokine production, avidity and proliferation. This will indicate not only whether patients have been successfully immunized, but also allow for a thorough immunological assessment of this genetic immunization strategy in human subjects.

To elucidate the mechanism by which this regimen generates T cell responses, we will analyze biopsies of AFP vector-injected and control intradermal sites as well as draining and contra-lateral lymph nodes. We hypothesize that the immunizing antigen (AFP) is cross-presented as protein to professional antigen presenting cells (APC) at the vaccine depot, which then traffic to draining lymph nodes to interact with T cells. We will test this hypothesis by analyzing draining sentinel lymph nodes and evaluating dendritic cells for vector sequences and their ability to stimulate AFP-specific T cell responses.

These studies will determine the clinical and immunological impact of a plasmid prime-adenoviral boost directed towards a self tumor antigen and to define mechanisms of antigen presentation.

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Keywords: hepatocellular cancer, alpha fetoprotein, immunotherapy

524 Focal Adhesion Kinase as an Immunotherapeutic Target for Prostate Cancer

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Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor tyrosine kinase involved in cancer progression and metastasis that is found overexpressed in a large number of tumors such as breast, colon, prostate, melanoma, head and neck, lung and ovary. Thus, FAK could be an attractive tumor associated antigen (TAA) for developing immunotherapy against a broad type of malignancies. In this study, we determined whether predicted T cell epitopes from FAK would be able to induce anti-tumor immune cellular responses.

To validate FAK as a TAA recognized by CD4 helper T lymphocytes (HTL), we have combined the use of predictive peptide/MHC class II binding algorithms with *in vitro* vaccination of CD4 T lymphocytes from healthy individuals and melanoma patients. Two synthetic peptides, $FAK_{143-157}$ and $FAK_{1000-1014}$, induced HTL responses that directly recognized FAK-expressing tumor cells and autologous dendritic cells pulsed with FAK-expressing tumor cell lysates in an HLA class II-restricted manner. Moreover, since the FAK peptides were recognized by melanoma patient's CD4 T cells, this is indicative that T cell precursors reactive with FAK already exist in peripheral blood of these patients.

Our results provide evidence that FAK functions as a TAA and describe peptide epitopes that may be used for designing T cell-based immunotherapy for FAK-expressing cancers, which could be used in combination with newly developed FAK inhibitors.

Keywords: immunotherapy, tumor antigens, therapeutic vaccines

525 Vaccine-Induced Antibodies Modulate Receptor Biology and Kill Tumors

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We are developing a series of recombinant viral based vectors that can serve as human vaccines, alone, or in prime boost strategies that are currently in late-stage preclinical studies or being tested in phase I clinical trials. These vaccine platforms build upon our prior experience with other vaccination strategies, including dendritic cell based immunotherapy, but appear to be potent stimulators of T and B cell responses, even in the presence of preexisting vector immunity. We have enhanced the vaccine efficacy using novel vector constructs and observed potent T cell and antibody-mediated immune responses in pre-clinical animal models. A major focus of these studies is the role of the tumor-specific antibodies induced by our vaccines (vaccine induced antibodies), which exhibit both classical immune mediated tumor cell killing, but also inhibit tumor proliferation, and alter receptor signaling. This novel insight into antibody mediated effecter mechanisms could improve our understanding of how immunotherapy impacts tumors and may lead to more effective cancer vaccines.

Keywords: vaccine, immunotherapy, viral vector

526 Phase II Clinical Trial in NSCLC With Lucanix™, a Cocktail of Four TGF-β2 Antisense Gene Modified Tumor Cells

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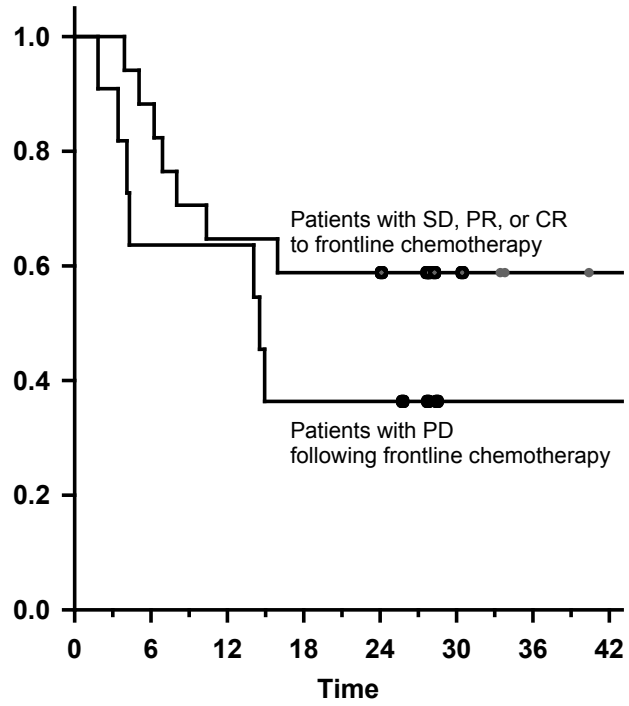
In a phase II clinical trial 75 patients were enrolled and treated with a therapeutic vaccine cocktail consisting of four TGF-β2 antisense gene modified allogeneic tumor cells. The patient population consisted of two stage IIB, 12 stage IIIA, 15 stage IIIB and 46 stage IV non-small cell lung cancer. Patients were randomized in three demographically balanced cohorts. Cohort 1 patients received injections with 1.25×10^7, patients in cohort 2 received 2.5×10^7 and patients in cohort 3 received 5×10^7 vaccine cells in monthly intervals. Dose-related survival differences were observed and statistical analysis showed that at doses $\geq 2.5 \times 10^7$ cells/injection there was a statistically significant improvement in survival, $p = 0.0151$, compared to the survival achieved by subjects in cohort 1. One year and two year survivals were 60 and 40 percent in cohorts 2 & 3 and 45 and 20 percent in cohort 1 respectively. Patients who had a clinical response to the vaccine therapy had a significantly higher level of IFN-γ production by peripheral blood mononuclear cells compared to patients who did not respond to therapy. Twenty-eight patients entered the trial having received one regimen of chemotherapy (±adjuvant), 17 patients with stable disease, a partial response, or a complete response to the chemotherapy regimen and 11 with progressive disease. The median survival of the patients who entered the trial with progressive disease was 14.5 months from the time of randomization (see the Kaplan-Meier survival curve below). The median survival of patients with stage III-IV disease who entered the trial with stable disease or better presented in the graph below was 44 months. These finding justified initiation of a Pivotal Phase III Clinical Trial with Lucanix™ as a maintenance therapy in patients who respond to frontline platinum based chemotherapy with stable disease, partial, or complete response.

Survival of Stages IIIA, IIIB, and IV NSCLC Based on Response to Frontline Chemotherapy



Keywords: non small cell lung cancer (NSCLC), Lucanix, TGF-β2 antisense

527 Translational Research Program in Dendritic Cell Biology and Therapy

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The goals of our program are to explore the ability of dendritic cell (DC) populations to elicit effective anti-tumor immunity by presenting tumor antigens to the immune system. We have carried out experiments that test vaccines in mouse models, in primates and in Phase I/II clinical trials. We have focused on pancreatic cancer and melanoma.

For pancreatic cancer, we have explored the ability of various forms of the tumor antigen MUC1 presented by DC to elicit effective anti-tumor immunity. We will present our results that show that in MUC1 transgenic mice, DC-loaded with the MUC1 tumor peptide, generate low level immunity due to poor expansion of MUC1 peptide-specific effector T cells and a more vigorous expansion of regulatory T cells (Tregs). On the other hand, MUC1 tumor glycopeptide is processed by DC into both peptides and glycopeptides leading to effective stimulation of glycopeptide specific effector T cells able to overcome the Treg function. All MUC1 vaccines tested to date have been based on the MUC1 peptide. We cannot be certain that MUC1 Tg mice faithfully represent how MUC1 antigen is handled by the cancer patient's immune system. For that reason, we have made synthetic forms of the rhesus macaques MUC1 peptide and glycopeptide and are in the process of immunizing monkeys with autologous DC loaded with MUC1. We are interested in knowing if in the primate model, the MUC1 glycopeptide is also more immunogenic.

We have already completed a clinical trial in pancreatic cancer patients of a DC/MUC1 peptide vaccine with very encouraging results. Twelve patients with early stage disease, whose tumors were removed, were given three vaccinations three weeks apart consisting of autologous DC loaded with MUC1 peptide. Five of these patients survived longer than four years and three are alive five years later with no evidence of disease. If the DC/MUC1 glycopeptide vaccine shows better immunogenicity in monkeys, as it did in mice, we will initiate a new trial using the glycopeptide.

In our studies in melanoma, we have explored the ability of DC to take up whole tumor cells and preferentially stimulate immunity against tumor specific antigens, known and unknown. We have completed enrollment. Adverse events have been limited to Grade 1 events such as a skin reaction at the injection site. We are now completing the analysis of immune responses.

An important extension of our studies is the development of strategies to target delivery of antigens and adjuvants to DCs *in vivo*. We have shown that cutaneous delivery of lentivirus results in direct transfection of DCs and potent and prolonged $CD8^+$ T-cell immunity. This immunization strategy induced strong T-cell responses against the melanoma-self antigen TRP-2, and a single immunization induced tumor specific immunity sufficient to inhibit tumor growth and prolong survival of tumor bearing animals.

Keywords: dendritic cells, vaccines, tumor antigens

528 Hematopoietic Cell Transplantation for Treatment of Hematologic Malignancy

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City of Hope/Beckman Research Institute

The City of Hope Program Project Grant conducts translational clinical research studies to address some of the major obstacles to successful autologous and allogeneic hematopoetic cell transplantation (HCT) for hematologic malignancies. The overall goals of this grant are to: 1. To improve the long-term, disease-free survival of patients with hematological malignancy, 2. To extend the potential benefits of HCT to older patients with leukemia and lymphoma by the development of more effective and safer transplant regimens, 3. To translate previous laboratory observations in virology and tumor immunology made in the City of Hope program into effective treatment strategies for patients undergoing HCT. Project I entitled "Radioimmunotherapy-based Transplant Regimens for Treatment of B-cell Lymphoma and Acute Myelogenous Leukemia" is focused on developing novel RIT-based preparative regimens in both the autologous and allogeneic setting. The goal is to improve anti-tumor potency and tolerability which can be tested in older patients with these diseases using radiolabeled antibodies to the CD20 antigen for treatment of lymphoma and radiolabeled antibodies targeted to the CD33 antigen in patients with AML/MDS. Project II entitled "Targeting Post-Transplant Minimal Residual $CD19^+$ ALL with Genetically Modified CD19-specific T-cells" will pilot adoptive therapy utilizing CD19-specific genetically-modified T cells as a strategy to target post-HCT minimal residual disease in patients with ALL. In this project we also answer important questions about the immunobiology (*in vivo* expansion, homing and targeting to sites of disease) of donor-derived T-cell adoptive transfer in recipients of allogeneic T-cell depleted HCT for $CD19^+$ ALL. Project III entitled "Vaccine Induced Immunity to CMV" will test the first CMV vaccine intended for PBSC donors for augmenting transfer of CMV immunity to the patient with the goal of limiting post-HCT viremia. This project is based on immunologic evaluation of proteins and peptides derived from the virus and will be tested by donor and recipient immunization to augment CMV-specific immunity.

Keywords: radioimmunotherapy, CMV vaccine, adoptive T cell immunotherapy

529 Genetically Enhanced Dendritic Cell-Based Immunotherapy for Cancer

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Despite the predominant role of dendritic cells (DCs) in priming immune responses to tumor- or pathogen-derived antigens, vaccines based on *ex vivo*-matured DCs have been almost universally disappointing, suggesting that a redesign of DC vaccines is called for. We recently demonstrated that prolonging DC survival or enhancing their activation state using chimeric costimulatory molecules could significantly improve *in vivo* expansion of tumor-specific T cells and anti-tumor responses. This was attributed, in part, to improved DC migration to lymph nodes and prolonged T cell interactions that favor Th1 development over alternative T cell differentiation programs. Our initial approaches relied on synthetic activation of an inducible allele of the co-stimulatory molecule CD40, iCD40, in antigen-pulsed DCs (HanksB et al (05) Nat Med 11, 130), or overexpression of a highly activated, lipid raft-targeted Akt allele, S*Akt (ParkD et al (06) Nat Biotec 24, 1581). Inducible alleles have the advantage over constitutive or systemic activation of proteins by permitting spatiotemporal control and the capacity for rapid reversibility, improving safety.

Subsequently, we observed that for optimum DC maturation, activation and anti-tumor efficacy, iCD40-expressing DCs needed to be treated with adjuvants, such as toll-like receptor 4 (TLR4) ligands. However, systemic application of TLR4 ligands can increase non-specific T cell responses or prohibitive pro-inflammatory responses. To circumvent the limitations of local or systemic adjuvant use and extend DC engineering to the next level, we have recently developed inducible alleles of pattern recognition receptors (i.e. TLRs, NOD2, RIG-I) and downstream signaling molecules (i.e. MyD88, TRIF) using a similar approach. We have also developed composite, unified vectors that contain both TLR and CD40 signaling. Moreover, we have developed tamoxifen-inducible versions of our potent Akt allele, termed inducible “super” Akt (iS*Akt) that can greatly extend DC accumulation in lymph nodes, promoting T cell expansion. These novel reagents should now permit targeted adjuvant stimulation of DCs *in vivo* following establishment of an immunological synapse instead of 1-2 days prior to synapse formation, as is standard practice.

In addition to describing these novel, and broadly applicable, reagents, our upcoming iCD40-DC-based, phase I/II (3+3), dose-escalation study against metastatic androgen-independent prostate cancer will be discussed along with the preclinical pharmacology and toxicology results utilizing the clinical reagents. If successful, these enhanced DC-based approaches should be applicable to both a wide variety of tumors, as well as exogenous pathogens.

Keywords: immunotherapy, prostate cancer, dendritic cells

530 Development of a Breast Cancer Vaccine Using Glycosylated and Anchor-Modified MUC1 Peptides

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The development of an effective vaccine for breast cancer is a high priority, as women with advanced stage cancer are at high risk of metastasis and relapse, even with optimal treatment. Cancer vaccines have the potential of controlling disease, prolonging time to recurrence, and ultimately even serving as a preventive measure. This project is focused on development of an effective tumor vaccine designed to elicit cytotoxic T lymphocytes (CTLs) against carbohydrate antigens, using MUC1 as the core glycopeptide. MUC1 is a widely expressed tumor-associated antigen that is over expressed and aberrantly glycosylated on greater than 90% of breast carcinomas and has been shown to elicit tumor-specific immunity. MUC1 occurs naturally as a heavily glycosylated protein which contains two well-known tumor-associated carbohydrate antigens (TACA): the disaccharide Thomsen-Friedenreich (TF) antigen (β-Gal-(1->3)-α-GalNAc-*O*-serine/threonine and its precursor, the monosaccharide Tn (GalNAc-*O*-serine/threonine). These glycans, which are widely expressed on breast and other tumors, are not often detected in normal tissues. Our hypothesis is that eliciting immunity to glycosylated and/or anchor-improved MUC1 peptides will result in anti-tumor immunity and long-term immune memory.

We have developed several short (9-mer) glycosylated and/or anchor-modified peptides derived from the MUC1 extracellular tandem repeat (TR) and cytoplasmic tail (CT) sequence. Native MUC1-peptides were altered by introduction of the monosaccharide, GalNAc-*O*-threonine (Tn carbohydrate) at the threonine in fifth position, and/or modification of amino acids in the major histocompatibility complex (MHC)-class I anchor positions. CTLs were generated against the modified HLA-A2 restricted peptides using peripheral blood mononuclear cells (PBMCs) from eight normal aged (peri- or post-menopausal) HLA-A2 individuals. The CTLs were effective in killing the target MCF7 cells that express MUC1 endogenously (HLA-$A2^+$) and MDA-MB-231.MUC1 cells transfected with MUC1 (HLA-$A2^+$). The CTLs were not lytic against MDA-MB-231.Neo cells that lack MUC1 expression. T cells from 35% of HLA-$A2^+$ breast cancer patients (n=23), chosen regardless of the pathological stage of their tumors, proliferated in response to the MUC1 peptides. These peptides are expected to be highly immunogenic in a clinical trial of breast cancer patients. The optimal peptides that have been selected are: SLAPPVHNV and SLAPT(Tn)VHNV. Based on considering 30% cell kill a response, it appears that at least 47% of donors receiving SLAPPVHNV and at least 35% of donors receiving SLAPT(Tn)VHNV will respond. The design of the clinical trial, which will compared the non-glycosylated peptide with the glycosylated peptide, is in progress.

In the meantime, a clinical trial funded by a Department of Defense Clinical Translation Award, is opening now, utilizing non-glycosylated peptides from MUC1 (class I) and HER-2/neu (class I and II). The phase I trial will enroll 45 patients who have completed adjuvant therapy for breast cancer (3 to 12 months post end of therapy). The peptides will be given with: Arm 1) GM-CSF, Arm 2) unmethylated CpG oligodeoxynucleotides ODN (PF-3512676 (formerly known as CpG 7909) or Arm 3) GM-CSF and CpG ODN). Of key importance in this trial are the participants, who are free of detectable disease and have likely recovered their immune function. Also of major importance is the examination of the immune effects of the CpG in humans; CpG has been shown to be highly effective in preclinical studies and is expected to promote DC, T cell and NK cell functionality in conjunction with the peptide vaccine. These clinical trials, utilizing peptides from important tumor antigens, should provide a strong foundation for the further development of effective cancer vaccines.

Supported by NCI/NIH P50 CA116201 Mayo Breast Cancer SPORE

Keywords: breast cancer, MUC1 vaccine, tumor-associated-carbohydrate-antigens (TACA)

531 Understanding Underlying Mechanisms and Optimizing Photodynamic Therapy (PDT)

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Photodynamic therapy (PDT) is a three component process involving a non-toxic, tumor-avid photosensitizer (PS), activating light and molecular oxygen producing singlet oxygen and other oxidative species that cause widespread cellular damage, alterations in signaling pathways and *in vivo* host responses. PDT induces cell death by apoptotic, necrotic and autophagic mechanisms. It also can shut down tumor vasculature, and induce host inflammatory and systemic immune responses. PDT is effective clinically, but the complex dosimetry and underlying molecular and tissue-level mechanisms are not well understood, nor has there been substantial investigation of rational combinations of PDT with agents targeting intracellular signaling pathways.

We have been designing and evaluating new PS including HPPH (2-1[hexyloxyethyl] -2-devinyl pyropheophorbide-a), a second generation agent optimized by *in vivo* structure-activity studies in preclinical models that now is in PII clinical trials for H&N, esophagus, and lung cancers. We also are developing multimodality agents combining fluorescence and/or PET-based imaging with tumor targeted PDT[1].

We found the ABCG2 transporter removes many clinical PS from tumor cells including cancer stem cells, but that transport is inhibited by TKIs such as imatanib mesylate (Gleevec); inhibition at the time of PS administration enhances *in vivo* PDT[2]. We plan PI/II clinical trials of effects of Gleevec with PDT. We also find that combining PDT with inhibitors of the cMET receptor and mTOR pathway enhances preclinical efficacy in cells and *in vivo*; this work will be extended to PI/II clinical trials. As discussed in the abstract by TH Foster, PS auto-oxidization (photobleaching) kinetics are a metric for efficiency[3]; and we plan PII clinical trials of topical ALA-PDT for superficial and nodular basal cell carcinoma (BCC) and in situ squamous cell carcinoma to utilize PS photobleaching rates to establish optimum irradiances and minimal pain, and then to extend the trials to determine the dose-response relationships and recurrence rates at these low irradiances.

The oxidative reactions in PDT and the multiple cell death pathways generate new epitopes and tumor associated antigens. In addition, the inflammatory reactions create a milieu that matures dendritic antigen presenting cells. Our investigation into the ability of PDT to enhance anti-tumor immunity led to the novel discovery that PDT-treated tumor cells are effective anti-tumor vaccines[4]. A pilot clinical study showed for the first time that PDT of BCC augments patient reactivity to a tumor associated antigen[4]. In preclinical models we found that ex vivo, PDT- generated vaccines can be used adjuvantly to induce systemic anti-tumor immunity. We are extending this finding to PI trials of autologous, PDT-generated *ex vivo* vaccines against melanomas, and also against BCCs in patients with nevoid basal cell carcinoma syndrome who develop 10's- 100's of carcinomas per yr.

References; [1]Pandey SK, et al. Med Chem. 48:6286-95, 2005. [2]Liu W et al. Clin Cancer Res. 13:2463-70, 2007. [3]W.J. Cottrell et al. Clin. Cancer Res. In press, 2008. 4Gollnick et al., Cancer Res. 62:1604-8, 2002. [5]Kabingu E, et al. in preparation.

Keywords: photodynamic therapy (PDT), ABCG2, anti-tumor immune responses

532 Dendritic Cells as Therapeutic Vaccines in Cancer

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Cancer immunotherapy seeks to mobilize a patient's immune system for therapeutic benefit. It can be passive (i.e., transfer of immune effector cells (T cells) or proteins (antibodies)) or active (i.e., vaccination). Early clinical trials testing vaccination with ex vivo-generated dendritic cells (DCs) pulsed with tumor antigens provide a proof-of-principle that therapeutic immunity can be elicited.

In our center between March 1999 and February 2005, sixty-four patients with metastatic melanoma were treated with DC vaccines in the course of four phase I/IIa clinical trials. DCs were generated either from CD34+ hematopoietic progenitors or from blood monocytes. Forty-nine HLA-A*0201$^+$ patients received vaccines pulsed with melanoma antigen derived peptides. Twenty-one patients received DCs loaded with killed allogeneic tumors regardless of their HLA type. Patients received up to eight vaccinations with antigen-loaded DCs over a maximum of seven months.

DC vaccinations were safe and tolerable. Nine of 64 patients were alive as of January 2008, from 39-105 months post vaccination. Median survival was 17 (95% CI 12-26) months. Preliminary analysis demonstrated the induction of long-lived melanoma-antigen-specific CD8+T cells in a patient who obtained durable complete regression of in-transit melanoma. DC vaccination expanded circulating MART-1-specific CD8+ T cells. These could be detected after the 4th and 8th DC vaccination as well as 2.5 years after the last vaccination with DCs. The T cells predominantly had an effector memory phenotype.

Yet, the clinical benefit measured by regression of established tumors in patients with stage IV cancer has been observed in a fraction of patients only. Two immune parameters appear linked to clinical outcome of the patients: 1) Objective clinical response is associated with induction of melanoma-specific effector cells; and 2) All patients display melanoma-specific IL-10-secreting CD4+T cells with regulatory/suppressor function that may counteract effector cells. Thus, we need to identify the next generation DC vaccines that are able to generate large numbers of high-avidity effector CD8$^+$ T cells as well as to overcome regulatory T cells and the suppressive environment established by tumors, a major obstacle in metastatic disease. Our pre-clinical studies actually demonstrate that Langerhans cells, a DC subset that is absent in IL-4-generated monocyte DCs, are superior in their capacity to prime high-affinity melanoma-specific CD8+T cells that are able to kill authentic tumor targets.

DC-based vaccination will become an essential component in cancer management. The considerable progresses made in the knowledge of DC biology as well as effector/regulatory T cell biology clearly open the avenues for development of considerably improved clinical protocols. These will include therapeutic vaccination of metastatic disease and preventive vaccination in patients with resected tumors. Therapeutic vaccination protocols will combine improved DC vaccines with chemotherapy to exploit immunogenic chemotherapy regimens. The ultimate ex vivo-generated therapeutic DC vaccine will be heterogeneous and composed of several subsets, each of which will target a specific immune effector. These ex vivo strategies should help to identify the parameters for DC targeting in vivo, which represents the next step in the development of DC-based vaccination. We foresee adjuvant vaccination in patients with resected tumors (but who have a high risk of relapse) to be based on in vivo-targeting of DCs with fusion proteins containing anti-DC antibodies, antigens from tumor stem/propagating cells, and DC activators.

Keywords: dendritic cells, therapeutic vaccines, melanoma

533 A Phase I/II Trial of Carbonic Anhydrase 9-Molecularly Targeted Kidney Cancer Vaccine Therapy

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The CA9-G250 kidney cancer vaccine translational research protocol represents the culmination of ten years of investigator initiated, bench to bedside pre-clinical research, and will be the final forum for the clinical testing of a GMP-quality vector that was manufactured following a highly competitive peer-reviewed grant application to the National Cancer Institute (NCI) Rapid Access to Intervention Development (RAID) program, and which was selected for presentation at the NCI Translational Science Meeting as an exemplar of NCI-funded translational research. A single center clinical trial of a novel vaccine targeting a common tumor protein in patients with metastatic renal cell carcinoma (RCC) is proposed. Approximately one third of RCC patients will have advanced disease at presentation, and one third of patients with localized disease will eventually progress to metastatic disease. Metastatic RCC poses a therapeutic challenge because of its resistance to conventional modes of therapy such as chemotherapy and radiation therapy. While it is clear that strides have been made in the struggle against metastatic RCC, the overall response rates of FDA approved therapies remain approximately 20% at best. Although, recent molecular targeted agents have recently been approved for RCC, these agents represent an incremental advance as disease progression is prolonged yet long term cures have not been achieved with these new therapies. Furthermore, these response rates are obtained at the cost of substantial toxicities to the patient. As a result, there remains an urgent need for the development of novel therapies to manage patients with metastatic or high risk, locally advanced RCC. **One such concept would be to develop an immune based therapy that is also molecularly targeted.**

The objective of this proposed clinical trial grant application, therefore, is to test a novel tumor vaccine developed in our laboratory that is capable of inducing a specific immune response against RCC tumors in patients who have been carefully selected as those most likely to respond to the experimental therapy. We propose using patients' own immune cells that have been genetically engineered to express a protein consisting of carbonic anhydrase IX (CAIX), a novel RCC associated protein that is expressed in almost all RCC tumors and which is linked to GM-CSF, a potent immune stimulator which is capable of amplifying a CAIX-specific immune response. Based on our preliminary data we believe that the fusion protein, GM-CAIX, will provide added benefit and potency as compared to the use of either agent alone. The clinical protocol consists of a phase I/II clinical trial of a GM-CSF-CAIX based vaccine therapy. This study will utilize vaccine material produced with Current Good Manufacturing Practices (cGMP) in joint collaboration with the National Cancer Institute (NCI) Rapid Access to Intervention Development (RAID) program, and will introduce the concept of patient selection using CAIX-based "molecular eligibility criteria." As CAIX is expressed by other solid tumors in the context of tumoral hypoxia, an effective CAIX-based vaccine could have wider oncologic applications than RCC alone.

Keywords: kidney cancer, vaccine, CAIX

534 From Benchtop to Bedside: Autologous Tumor-Derived Heat Shock Proteins Can Evoke a Tumor-Specific Immune Response in Patients With High-Grade Glioma

Andrew Parsa and UCSF Brain-SPORE Investigators

Background: Autologous heat Shock Proteins (HSPs) derived from cancer cells have successfully evoked tumor specific immune responses in animal models and cancer patients. Here we describe work that originated from a career development award to the UCSF SPORE in 2002, evolved into a clinical trial in 2005 and subsequently an independent project for the UCSF Brain SPORE renewal in 2007.

Methods: Based on preliminary work through career development awards a Phase 1/2, open-labeled study designed to evaluate the safety, feasibility, best tolerated dose, and preliminary efficacy of HSPPC-96 (Oncophage®) in twelve patients with recurrent glioma. Peripheral and local immune responses were also evaluated.

Results: HSP vaccine was well tolerated with no serious adverse events attributable to investigational therapy in 12 Phase I patients. Overall median survival approached 42 weeks post-resection. The majority (11/12) of patients survived beyond 26-weeks, with 4 of 12 patients survived beyond 48 weeks. All patients exhibited a significant innate immune response following vaccine administration and 11 of 12 patients demonstrated CD8 T cell IFN gamma production upon restimulation with recombinant gp-96 ($p<0.01$). Additionally, the majority of patients (11/12) exhibited significant Th1 type responses as measured by multi-cytokine qPCR. The presence of an adaptive immune response and minimal residual disease was associated with survival beyond 36 weeks.

Conclusions: Heat-shock protein vaccines are safe for patients with recurrent glioma and appear to evoke a specific and innate immune response associated with overall survival. Further study in a Phase 2 setting is warranted. The SPORE funding mechanism has allowed the transition of this technology from the benchtop to bedside for glioma patients. Additionally, the SPORE mechanism has provided the infrastructure to facilitate the acquisition of 3 NRSA post-doctorial grants, and a Howard Hughes Medical Institute training grant directly related to the results described.

Keywords: glioma, vaccine, heat shock proteins

535 Enhanced CD8 CTL Cross Priming as Vaccine Principle

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Gp96 is the one of the major protein and peptide chaperones of the endoplasmatic reticulum. It helps folding of membrane associated and secreted proteins and transports peptides on their way to MHC class I presentation. Srivastava was the first to show that gp96 isolated from tumor cells and injected into syngeneic mice was able to induce a tumor specific immune response that was able to protect mice from a subsequent challenge with the same tumor but not against other tumors. Gp96 is taken up by dendritic cells and macrophages via its endocytic receptor CD91, resulting in the activation of DC, independent of CD4 cells and CD40-L/CD40 interaction. Uptake of gp96 and its chaperoned peptides results in cross presentation of the peptides by MHC I of the DC and priming of antigen specific, cognate CD8 T cells with over one million fold higher efficiency than intact protein.

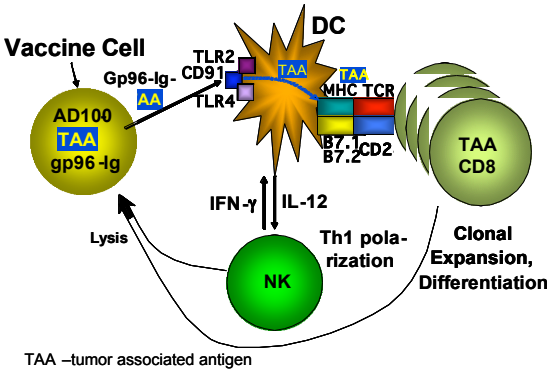

Figure 1: Putative activation of CD8 CTL by gp96-Ig-peptide

To make gp96 suitable in a vaccine system with allogeneic cells we genetically modified the protein by deleting its endoplasmatic reticulum retention signal and replacing it with the Fc portion of IgG1. Gp96-Ig transfected tumor cells secrete gp96-Ig along with its chaperoned peptides. Injection of gp96-Ig transfected tumor cells into syngeneic mice results in tumor rejection associated with the clonal expansion of cognate CD8-CTL to a frequency of 15-40% of all CD8 cells (4). Clonal CD8 CTL expansion is enhanced in CD4 deficient mice while CD40-L deficient mice are similar to w.t. mice. The combined knock out of B7.1 and B7.2 completely abrogates CD8 expansion while the loss of either single gene reduces expansion to about 50% of w.t. NKT cells are not required but NK cells enhance CD8 CTL responses. Antigen cross presentation and cross priming of cognate CD8 cells is ~20 million fold enhanced when peptides are chaperoned by gp96 compared their presence as intact protein, as measured with ovalbumin TCR transgenic system. Importantly, gp96-Ig mediated antigen specific cross priming works efficiently in the absence of lymph nodes in LTα deficient mice.

Following the events after intraperitoneal injection of tumors secreting gp96-Ig, we found that gp96-secretion promotes recruitment of monocyte/macrophages, dendritic cells and NK cells. DC and NK cells become activated only when gp96 is secreted by the injected tumor cells leading to CD8 CTL expansion initially locally followed by systemic expansion. Lymphotoxin α k.o. mice have no peripheral lymph nodes including mesenteric lymph nodes and Peyer's patches, however they are able to support gp96-Ig mediated CD8 CTL expansion to a similar extent as wild type mice. Using the ovalbumin as surrogate antigen, alllogeneic tumor cells transfected with ovalbumin and gp96-Ig are able to mediate cognate CD8 CTL (OT-I) expansion to the same extent as syngeneic cells. Gp96-Ig deliverd by allogeneic tumor cells thus has many properties that make it suitable for vaccine purpose.

We have cotransfected the AD100 NSCLC line with HLA A1 and gp96-Ig. One million cells secrete 400ng gp96-Ig within 24h in tissue culture. Upon irradiation with 12,000 rad the cells continue to secrete gp96-Ig at declining rates for the next two weeks in culture. In the phase I study that has just opened we will enroll 24 patients with stage IIIB/IV NSCLC who have failed at least two lines of prior therapy. Early results will be presented and further vaccination strategies discussed.

Keywords: lung cancer, chaperones, CTL

536 Replicating Adenovirus-HIV Recombinants Provide a Comprehensive Vaccine Approach

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As HIV is transmitted primarily across mucosal barriers, adenoviruses (Ad) are vectors of choice for an HIV vaccine. Ad infect epithelial cells at mucosal inductive sites, eliciting mucosal and systemic cellular and humoral immunity. In general, live, replicating vaccines (e.g. smallpox, yellow fever, measles) have been most successful, providing life-long immunity. Therefore, we have developed replication-competent Ad-recombinant vectors. We established that replicating, compared to matched non-replicating Ad-HIV recombinants, elicit better cellular immune responses and prime higher titer antibodies able to neutralize primary HIV isolates and mediate antibody-dependent cellular cytotoxicity (ADCC) across a spectrum of HIV clades (Peng et al., J Virol, 2005; Gomez-Roman et al., JAIDS, 2006). Further in the SIV rhesus macaque model, potent, durable protection against a mucosal challenge was achieved using Ad-SIV prime/SIV envelope protein boost regimens (Patterson et al., J Virol, 2004; Malkevitch et al., Virology, 2006). Particular combinations of Ad-recombinants encoding different HIV antigens have also led to apparently synergistic protective efficacy (Demberg et al., J Virol, 2007).

Recently, we compared sequential intranasal/oral (I/O) and oral/oral (O/O) priming with Ad-SIV vaccines followed by SIV envelope boosts and an intrarectal SIV_{mac251} challenge (Zhou et al., Vaccine, 2007). The I/O regimen elicited better systemic cellular immunity, but evaluation of peripheral blood by standard ELISPOT and proliferative assays did not reflect mucosal immune responses. Both regimens induced SIV-specific T cells expressing gut homing receptors and memory T cells at mucosal effector sites. Further, both regimens gave equivalent protection at the set point of SIV infection. Nevertheless, the I/O regimen conferred significantly better acute phase protection than the O/O. To elucidate the basis for this difference, we evaluated systemic and mucosal antibody responses. Neutralizing antibodies against primary SIV_{mac251} were not detected, but potent antibodies mediating ADCC and antibody-dependent cell-mediated viral inhibition, both correlated with reduced acute viremia, were induced. Importantly, antibodies in rectal secretions that mediated inhibition of transcytosis across epithelial cells were significantly higher in the I/O compared to the O/O group (Hidajat et al., in preparation). Our results suggest a spectrum of systemic and mucosal functional antibodies contribute to acute phase protection.

Overall, our findings illustrate the comprehensive nature of the replicating Ad-recombinant prime/protein boost strategy. All immune system components are engaged and contribute to protection. The demonstrations of strong immunogenicity and potent protective efficacy have moved this approach forward to phase I human trials. Based on established safety and efficacy of wild-type Ad4 and Ad7 oral vaccines in US military recruits, we selected an Ad4 vector for clinical development. Safety and immunogenicity of an Ad4-HIV*env* vaccine given by oral tablet will be tested in HIV seronegative volunteers. Production of a GMP vaccine lot is underway. In addition to enhanced immunogenicity, the oral replicating vaccine has practical advantages. It is dose sparing, does not require freezer storage, and is easily administered for worldwide use.

Keywords: HIV vaccine, replication-competent adenovirus vectors, mucosal immunity

537 Polymeric L2 as an Inexpensive and Broadly Protective Human Papillomavirus Vaccine

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Current preventive Human papillomavirus (HPV) vaccines based upon L1 virus-like particles (VLPs) target only two oncogenic HPV genotypes although cervical cancer develops from persistent infection by any of 15+ oncogenic HPV types. Over 85% of cervical cancer cases worldwide occur in Developing nations and the licensed vaccines currently cost over $300. Therefore, low cost and broadly protective HPV vaccines are urgently needed. We recently discovered that vaccination with the N-terminus (e.g. residues 11-200) of the L2 minor capsid protein of HPV can produce broadly cross-neutralizing antibodies and protect against divergent papillomavirus type infections in the rabbit and mouse challenge models. However, serum neutralizing antibody titers and protection against experimental challenge were greater for homologous type virus as compared to heterologous type papillomavirus. Thus, while vaccination with L2 induces antibodies that cross-neutralize diverse papillomavirus types, L2-specific antibodies typically neutralize related types more effectively than less evolutionarily related types. To provide the broadest possible immunity with a single vaccine antigen, we are using a “Beads on a String” approach, e.g. a concatenated fusion polypeptide consisting of L2 11-200 peptides from HPV6, HPV16 and HPV18 (called L2 11-200x3), to induce higher levels of cross-neutralizing antibodies across a broader range of HPV types than the L2 polypeptide of any single type. Polymeric L2 proteins are less immunogenic than L1 VLPs. Therefore we have tested several adjuvants appropriate for clinical use for formulation with polymeric L2 vaccines. We find that a single cross-reactive antigen based upon the minor capsid protein L2 represents a possible inexpensive alternative to highly multivalent L1 VLP vaccines for broad protection against infection with high risk HPV types. With support from the NCI RAPID program, we are currently optimizing the purification of our polymeric L2 vaccine antigen from recombinant *E. coli* and its formulation with alum adjuvant for early phase clinical trials of this candidate preventive HPV vaccine.

Keywords: human papillomavirus, cervical cancer, prophylactic vaccine

538 EGFRvIII-Targeted Vaccine (CDX-110) Induces Immune Responses and Prolongs TTP When Given With Simultaneous Standard and Continuous Temozolomide in Patients With GBM

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Background: Conventional therapies for GBM fail to target tumor cells exclusively. Immunologic targeting of tumor-specific gene mutations may allow more precise eradication of neoplastic cells. The epidermal growth factor receptor variant III (EGFRvIII) is a consistent and immunogenic mutation that is not expressed in any normal tissues, but is widely expressed in GBMs and other neoplasms making it an attractive target for active immunotherapy.

Methods: A phase II multi-center clinical trial was undertaken to assess the immunogenicity and efficacy of an EGFRvIII-specific peptide vaccine in patients with newly-diagnosed, EGFRvIII+ GBM in combination with simultaneous standard or continuous temozolomide (TMZ). After resection and radiation/TMZ (75mg/m^2/d), consecutive cohorts received subsequent monthly cycles of 200 mg/m^2 (N=13) or continuous 100 mg/m^2 (N=8) TMZ simultaneous with intradermal vaccinations with an EGFRvIII-specific peptide (PEPvIII) conjugated to keyhole limpet hemocyanin (KLH) until tumor progression or death.

Results: 21 patients were enrolled. There was one allergic reaction, but no other SAEs. There were no significant differences in vaccine immunogenicity (P>0.999; binomial proportions), PFS (P=0.7979; logrank), or OS (P=0.7728; logrank) between TMZ regimens. Although TMZ induced Grade II lymphopenia in 53.8% of patients, the co-administration of TMZ with the EGFRvIII vaccine (CDX-110) results in strong sustained immune responses to EGFRvIII in 100% (CI_{95}: 0.72, 1.00) of evaluated patients. Median PFS was 16.6 months (CI_{95}: 9.1, 22.7). Median survival has not been reached. The survival of the vaccinated patients is better than a matched historical control group (14.3 months; CI_{95}: 13.0, 16.2)(P<0.0001; log-rank) and a subgroup treated with TMZ (15.2; CI_{95}: 13.9, 20.5)(P=0.0078) and is also equivalent to the results seen in patients vaccinated without simultaneous temozolomide (P=0.4108; logrank)

Conclusions: CDX-110 peptide vaccination with standard of care temozolomide in patients with GBM appears very promising, and is under investigation in a phase III, randomized clinical trial.

Keywords: chemoimmunotherapy, clinical trials, epidermal growth factor receptor (EGFR)

539 Development and Assessment of Interventions to Prevent Oncogenic HPV Infection

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Infection by one of fifteen mucosal HPV types is the central cause of cervical cancer and is also an important risk factor in a substantial fraction of anal, penile, vulvar, vaginal and oral cancers. The recently licensed HPV prophylactic vaccines are based on our L1 virus-like particle (VLP) technolog, and include VLPs of the two most oncogenic types,16 and 18. In phase III clinical trials, the vaccines were virtually 100% effective at preventing high grade cervical cancer precursor lesions induced by HPV16 or 18. Because protection from infection and cervical neoplasia was HPV type-restricted, the expectations are that VLP vaccination will reduce a women's risk of cervical cancer by 70-80%. While these vaccines represent a milestone in cancer prevention, they have several inherent limitations as public health interventions. Working with NCI, academic, and corporate partners, we are addressing these limitations by developing second generation prophylactic vaccines and other interventions to prevent oncogenic HPV infection. To expand protection to all HPV types via a monovalent vaccine, we are developing vaccines based on the L2 minor capsid protein, which, unlike L1 VLPs, induces broadly cross-neutralizing antibodies. To reduce the cost of vaccine production and administration in low resource settings, we are developing live oral *Salmonella typhi*-based L1 vaccines that might prevent both typhoid fever and cervical cancer. To complement prophylactic vaccines, we have identified carrageenan, an inexpensive gelling agent widely used in food and some sexual lubricants, as a very potent and broad spectrum genital HPV microbicide. We have demonstrated it's in vivo efficacy in mouse and macaque cervicovaginal challenge models developed in our laboratory. Using the macaque model, we have also observed that collection of a standard Pap smear specimen dramatically potentiates HPV infection of both the ectocervix and the endocervix. However, simply replacing the commonly used glove lubricating gel with a carrageenan gel for the subsequent bimanual pelvic exam reversed this potentiation. Phase I clinical trials of these three HPV infection prevention measures are in the planning stage.

Keywords: vaccine, microbicide, papillomavirus

540 GMP-Grade KLH Carrier Protein for Therapeutic Vaccines

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Stellar Biotechnologies, Inc.

Keyhole Limpet Hemocyanin (KLH) is a highly effective T-dependent carrier protein for induction of immune responses via MHC Class II presentation on antigen presenting cells. KLH conjugates of tumor antigens are capable of eliciting potent antibody and cell-mediated anti-tumor immune responses, and a number of therapeutic vaccines based on KLH conjugates are in clinical trials for lymphoma, melanoma, breast and other cancers. The growth of therapeutic vaccine research is rapidly increasing the demand for GMP-grade KLH; however, the source animal for KLH (the Pacific gastropod mollusk *Megathura crenulata*) is a fragile and sparsely distributed species that is acknowledged to be unlikely to survive commercial harvesting at the scale required for pharmaceutical products. Furthermore, there is a need for consistently high quality, stable, GMP-grade KLH for reliable conjugate vaccine manufacturing.

To address the growing need for sustainable supplies of GMP-grade KLH for vaccines, the first goal of our research has been the development of aquaculture technologies for *M. crenulata* as a means of raising KLH production animals and alleviating the vaccine community's dependence upon the wild resource. We have developed hatchery production methods for induction of gametogenesis in broodstock animals, and for spawning, fertilization, and induction of metamorphosis in *M. crenulata* larvae. Our work on larval *M.crenulata* is now focused on increasing the survival of post-larvae at this vulnerable stage of the life cycle in order to increase hatchery yield. We have also developed specialized diets and cultivation systems for adult animals, and shown that KLH from animals that are raised on these diets and in these systems is essentially identical in isoform distribution and other key physical characteristics to KLH from animals found in the ocean.

The other goal of our research has been to develop methods for processing and testing of high-quality KLH according to cGMPs for vaccines. A major accomplishment in this area has been the development of a stable, dissociated subunit form of KLH for use in conjugate vaccines. This lower molecular weight KLH forms smaller complexes than native KLH, resulting in higher solubility conjugates. An extensive battery of analytical and safety assays has been developed to support the quality control testing and stability testing of the product. Stability testing indicates adequate stability for a commercial product. A Drug Master File for the subunit KLH product is being prepared for submission to the FDA.

The availability of high-quality GMP-grade KLH will facilitate the translation of pre-clinical vaccine research into the clinic. Vaccine researchers can now formulate and test KLH conjugates with the assurance that successful pre-clinical conjugate vaccines can be carried into clinical trials using the same manufacturing methods, and with a minimum of re-formulation and testing.

Keywords: KLH, hemocyanin, antigen

541 Epithelial Ovarian Cancer: The Role of MUC16/CA125

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Sloan-Kettering Institute for Cancer Research

Despite advances in treatment, epithelial ovarian cancer remains the fourth leading cause of cancer death in american women. This program, now in its 15th year, has focused on the immunology and management of epithelial ovarian cancer. In the history of this grant, the program was responsible for the cloning of the MUC16/CA125 antigen, the development of a series of monovalent cancer vaccines directed at ovarian cancer epitopes and a number of studies related to consolidation therapy of patients in complete remission. Clinical trials of combination therapy in the primary treatment setting and for recurrent disease were also completed. Goals for the current funding period are 1. To examine the role of MUC16 in the pathophysiology of ovarian cancer and develop the scientific basis for possible MUC16 based treatment opportunities; 2. To develop both humoral and cellular strategies for ovarian cancer treatment to be used in the minimal residual disease setting, emphasizing MUC16 as a target; 3. To clinically test adoptive T cell based immunological therapies in Phase I pilot studies and in carefully designed definitive Phase II studies with appropriate correlative studies; and 4. To develop and test platinum based combinations with novel agents in re-induction. Each of the 4 projects in this competing renewal is directed at one or these goals. In a continuing project, we extend our studies of MUC16/CA125 into the genetic structure of MUC16 and the role expression of MUC16 has in the malignant phenotype. A second continuing project completes our examination of antibody vaccines and initiates a new strategy for the development of T-cell vaccines, based in part on the MUC16 sequence. A new project will investigate the in vitro amplification of tumor antigen specific cytotoxic T cells for adoptive therapy, employing HLA restricted epitopes from WT1 and MUC16. In the last continuing project, a clinical program will focus on re-induction of second remission with novel platinum based combinations and consolidation therapy for patients with minimal residual disease. Models of CA125 behavior are also explored in this project. Three themes unite these projects including the structure of MUC16/CA125 antigen and its biology; consolidation therapy as a novel strategy in ovarian cancer and a commitment to the development of immunologic therapies for ovarian cancer.

Specific Translational Outcomes from this program include correlative science investigations and multiple Phase I and Phase II trials conducted at MSKCC. These trials have been collated into a strategic clinical trials approach to both the first relapse and second complete remission. We are also implementing through the GOG a Phase III randomized trial of a polyvalent vaccine in patients in second remission.

Funding: 5PO1CA052477-15.

Keywords: vaccine, ovarian, mucin

542 Immune Therapies for HPV Disease: Lessons Learned From Phase I

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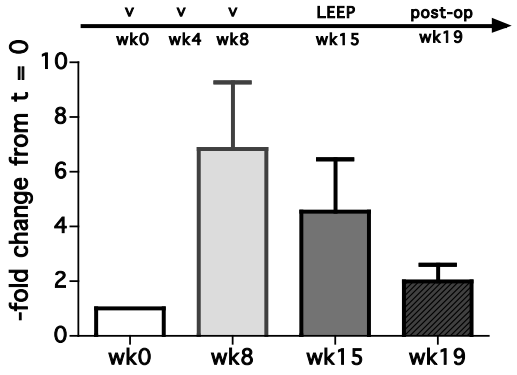
Human papillomavirus (HPV) causes 10% of malignancies in women. Despite the availability of screening and prophylactic vaccines, because barriers to access are significant, the need to pursue therapeutic strategies remains real. Globally, one genotype, HPV16, is associated with over half of all cervical malignancies. High grade cervical dysplasia, CIN2/3, the lesion which is the immediate precursor to invasive disease, is associated with integration of the HPV genome into the host genome, with subsequent constitutive expression of the viral oncoproteins E6 and E7, both of which play critical functional roles in disease initiation and persistence. Nonetheless, between 20-25% of established HPV16-associated CIN2/3 lesions undergo complete spontaneous regression, which is presumably immunologically mediated. This patient cohort presents an opportunity to determine immunologic parameters of disease outcome in incipient malignancy caused by a common, persistent viral infection. Successful immune therapies would obviate the need for surgery. We developed an HPV16 E7-targeted DNA vaccine which showed immunogenicity and antitumor effect in preclinical experiments. We developed GMP reagent in collaboration with NCI RAID, and carried out Phase I evaluation in otherwise healthy women with HPV16-associated CIN2/3.

The trial was a standard 3+3 dose-escalation design, with an expanded cohort at the highest dose. Healthy adult women with a biopsy-confirmed diagnosis of HPV16+CIN2/3 underwent three intramuscular vaccinations (0.5 mg, 1 mg, or 3 mg) of a plasmid expressing a Sig-E7Δ-HSP70 hybrid antigen on days 0, 28, and 56, followed by standard therapeutic resection of the cervical squamocolumnar junction at day 105 (week 15). Safety and immunogenicity of the vaccine, and histologic outcome based on resection at week 15 were assessed.

Vaccination was well-tolerated: no dose-limiting or severe adverse events were reported. Three of nine patients in the highest dose cohort had complete histologic regression of lesions at the time of resection. Although this rate of regression is slightly higher than what would be expected in an unvaccinated cohort, the difference is not significant. T cell response to vaccine antigen, E7, measured in the peripheral blood, did increase subsequent to vaccination in 8/15 subjects. However, while it was possible to elicit HPV-specific immune responses in subjects with established high grade dysplasia, this effect was transient. Because cervical lesions are relatively accessible, we developed methods to assess the local microenvironment, in both fresh and banked specimens.



Because other investigators have identified a significant immunogenic effect in humans specifically when DNA vaccination is used as a priming immunization in sequence with subsequent boost immunization with viral vectors, we tested heterologous prime-boost vaccination, first with an MVA construct to demonstrate proof of principle in our model. We have subsequently obtained a recombinant vaccinia construct which targets HPV16/18 E6/E7, as a kind gift from Celtic Pharma. The next phase of clinical testing will assess safety, immunogenicity, and clinical effect of heterologous DNA-prime-TA-HPV boost vaccination, either with or without a topical immune response modulator, in a CIN3 cohort.

Keywords: human papillomavirus (HPV), vaccine, intraepithelial neoplasia

543 Phase I Adjuvant p53 Based Immunotherapy for Head and Neck Cancer

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Cancer vaccines often target shared human tumor antigens, most of which are proteins overexpressed in tumors relative to normal cells. Mutation of the tumor suppressor protein, p53, is one of the most common events in human cancers. Furthermore, the direct involvement of p53 in the malignant transformation of tumors makes it an attractive target for immunotherapy. Most mutations in p53 are associated with accumulation of mutant p53, which can enhance the processing and presentation of p53 peptides for immune recognition. The resulting peptides are mainly wild type (wt) in sequence and represent non-mutated "self" epitopes.

We have demonstrated *ex vivo*, that stimulation of PBMC from healthy donors and subjects with head and neck cancer with "optimized" p53 peptides can circumvent their tolerance to the wt p53 peptides and induce detectable levels of anti-p53 cytotoxic T lymphocytes (CTL). Recent data indicate that multi-epitope cancer vaccines should incorporate $CD8^+$ T cell-defined peptides as well as $CD4^+$ T cell-defined peptides that activate CTL and $CD4^+$ T helper (Th) cells respectively. It is currently uncertain whether the CTL and Th peptides need to be derived from the same protein. Thus our approach incorporates peptides capable of inducing anti-p53 CTL as well as anti-p53 Th cells.

We have conducted a 3-arm phase I, adjuvant vaccine trial in head and neck cancer patients with no evidence of disease. The trial has accrued 15/24 patients to date. It employs autologous dendritic cells (DC) loaded with two "optimized" HLA-A2 binding peptides, namely the p53 T150L and F270W peptides, which target the CTL-defined wt $p53_{149\text{-}157}$ and $p53_{264\text{-}272}$ epitopes. The first arm received DC loaded with these two peptides alone, while the second and third arms also received DC loaded with the HLA-DR binding, T helper peptide $p53_{110\text{-}124}$ or a tetanus T helper peptide. Immunologic monitoring indicates enhancement in anti-p53 CTL frequencies and cytokine secretion. Continued accrual and immunologic assessment is ongoing and will be reported for the 15 vaccinees.

Keywords: cancer vaccine, head and neck cancer, p53

544 IL-2-Based Combination Immunotherapy Coordinates Innate and Adaptive Immune Responses for the Improved Treatment of Mouse and Human Renal Cell Carcinoma

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Immunotherapy against renal cell carcinoma (RCC) and other tumors requires the coordinated destruction of tumor cells, uptake and presentation of tumor antigen(s) and support for T cell effector and memory function. Interleukin (IL)-2 is an immune stimulating cytokine that promotes anti-tumor responses in preclinical RCC models and the clinic. Although IL-2 is an effective anti-tumor treatment, combination therapies of IL-2 with either IL-12 or anti-CD40 may be more effective. IL-2/IL-12 combinations elicit anti-angiogenic effects and endothelial cell apoptosis within the tumor vasculature. These events occur early upon IL-2/IL-12 therapy and are dependent upon host IFNγ, Fas and FasL expression. The resulting tumor destruction may result in the increased availability of tumor-associated antigens for more efficient immune targeting of the developing tumor.

Currently, IL-2/ IL-12 treatment is being evaluated in a Phase I clinical trial on adults with a variety of solid tumors, including RCC (19 of 24 patients). Six patients (25%) exhibited stable disease for at least 3 cycles and 2 patients (8%) had stable disease for >1 year. Partial responses (45-50% tumor regression) were observed in 2 patients and a minimal response was observed in 1 patient. Patients responding to therapy had an increased number of $CD4^+$ T cells and $CD56^+$ NK cells, elevated serum IFNγ levels and a decrease in tumor blood flow.

In addition to IL-12, IL-2 in combination with agonistic anti-CD40 has also proven to be an effective combination against preclinical RCC. CD40 ligation potently activates antigen-presenting cells to present tumor antigen(s) and produce cytokines, including IL-12. Similar to IL-2/IL-12 therapy, IL-2/anti-CD40 combination therapy resulted in synergistic anti-tumor responses that were dependent upon host IFNγ and FasL expression. Furthermore, IL-2/anti-CD40 combinations induced significant infiltration into the tumor by $CD8^+$ T cells, NK cells and macrophages. The expression of IFNγ and nitric oxide by these effector leukocytes was synergistically enhanced by IL-2/anti CD40 treatment.

Taken together, IL-2/IL-12 and IL-2/anti CD40 elicit synergistic anti-tumor responses through the enhanced recruitment and activation of immune cells within the tumor microenvironment. Therefore, combination strategies may be more effective than single agents for the treatment of metastatic RCC and other solid tumors.

Keywords: immunotherapy, cytokine, IL-2

545 Clinical Development of DNA Vaccines for Melanoma

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Immunity to self antigens on cancer is largely constrained by immunologic ignorance and tolerance. Pre-clinical studies in C57BL/6 mice have shown that injection of plasmid DNA vaccines encoding xenogeneic orthologues of melanocytic differentiation antigens results in the induction of antibody and T cell responses capable of protecting mice from challenge with syngeneic B16 melanoma. Creation of epitope enriched DNA vaccines in which the syngeneic antigen has been altered to create better MHC class I binding elements also results in potent CD8+ T cell responses and tumor protection. Based on these studies, a series of phase I clinical trials was conducted to assess the safety and immunogenicity of various DNA vaccines in patients with high-risk melanoma in the adjuvant setting. In parallel, a program in veterinary oncology was launched in which pet dogs with spontaneously arising melanoma were treated with xenogeneic DNA vaccines. A small pilot canine trial showed that life expectancy of dogs with WHO stage III and IV melanoma could be increased thee-fold with the use of xenogeneic tyrosinase vaccines, compared with historical controls. An additional 300 canine patients have been accrued and as a result of a collaboration with Merial, the human tyroinase DNA vaccine has become the first licensed (USDA) therapeutic cancer vaccine in the US. We have completed accrual and immunologic analysis of phase I trials using mouse and human tyrosinase and gp100 DNA vaccines in human melanoma patients. Using validated assays developed in our immune monitoring core, approximately 40% of patients have quantifiable CD8+ T cell responses to epitopes in the respective human antigen. GM-CSF DNA was investigated as a molecular adjuvant and shown to be capable of inducing CD8+ T cell responses with gp100 and tyrosinase peptides. Currently, clinical trials of TYRP2 DNA vaccines and a comparison of particle-mediated delivery versus intramuscular injection of gp100 DNA are ongoing. Based on the collective results of these early phase I trials, we plan to construct a polyvalent DNA vaccine with GM-CSF DNA as an adjuvant. The route of administration will be determined by the results of ongoing comparative studies of particle mediated epidermal delivery as well as a phase Ia/Ib trial of electroporation-assisted delivery of xenogeneic DNA vaccines.

Keywords: DNA vaccine, melanoma, Phase 1

546 Proposal for a Phase I/II Clinical Trial of a Therapeutic HER-2/neu Expressing Adenoviral Vector Dendritic Cell Vaccine That Cures Large Established Breast Cancers and Lung Metastases in Mice

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It has been notoriously difficult to treat large established tumors (>1cm^2) in pre-clinical animal models of cancer. In addition, despite the induction of tumor-specific T cell responses, cancer vaccines have rarely led to clinically significant tumor regression or cure in human clinical trials. HER-2/*neu* (ErbB-2/neu) is a protooncogene member of the EGFR family that is over expressed in 25-30% of breast cancers and is associated with clinically aggressive disease and a poor prognosis. Trastuzumab (Herceptin®) is a monoclonal antibody directed against HER-2/*neu* and has documented efficacy when combined with cytotoxic agents in the adjuvant treatment of metastatic HER-2/*neu* positive breast cancer. However, traztuzumab therapy has been associated with cardiac toxicity as well as non-responsiveness and the emergence of resistance. Alternative agents directed at this therapeutic target are needed and vaccines that elicit antibodies (in contrast to cellular responses) to HER-2/*neu* are currently not available.

The TUBO tumor model is a BALB/c murine mammary carcinoma that spontaneously arose in a BALB-neuT transgenic mouse expressing the rat neu oncogene. The tumor grows in subcutaneous or lung metastatic sites in BALB/c mice and will kill the animals if left unchecked. Our group has demonstrated that vaccination of BALB/c mice with an adenoviral vector expressing the extracellular (EC) and transmembrane (TM) domains of the rat ErbB2/neu oncogene (Ad-neuECTM) causes regression of large (>1cm^2) established subcutaneous mammary TUBO carcinomas and cures breast cancer lung metastases. The effect of Ad-neuECTM is antibody-mediated and does not require CD8 cells. In contrast to trastuzumab, vaccine-induced antibody is not dependent on FcRs and directly inhibits ErbB2 function and the growth of tumor cells. Polyclonal antibodies elicited by vaccination may target multiple HER-2/*neu* epitopes and be less susceptible to escape mutations or be effective in patients who have failed trastuzumab. In addition continuous antibody production following vaccination eliminates the need for repeated expensive monoclonal antibody administration.

We are proposing to conduct a Phase I/II clinical trial of an autologous dendritic cell (DC) vaccine transduced with an adenoviral human HER-2/*neu* ECTM vector in patients with metastatic HER-2/*neu* breast cancer who have failed prior therapy. Transduction of autologous DCs will result in prolonged and maximal expression of the HER-2/*neu* antigen and minimize the impact of pre-existing adenoviral immunity on generation of vaccine-induced responses. Given the unknown potential cardiac toxicity of vaccine-induced antibodies, we proposed to conduct the initial phase I trial in patients with 2+/indeterminate HER-2/*neu* expression to establish vaccine safety, toxicity and preliminary immunogenicity. Once safety data regarding cardiac toxicity have been documented, a phase II study of a selected vaccine dose will be studied in breast cancer patients with 3+ HER-2/*neu* expression that are disease free after surgery and adjuvant therapy or who have failed treatment with trastuzumab.

Reference: Park JM et al. Cancer Res 2008;68(6):1979-87.

Keywords: HER-2/*neu*, adenoviral transduction, dendritic cell vaccine

547 Development of Antigen-Targeted Vaccines and Immune Checkpoint Inhibitors for Cancer Therapy

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T cells represent critical immunologic effectors in the anti-tumor immune response produced by cancer vaccines aimed at both cancer treatment and prevention. We have been utilizing current molecular technologies to develop novel approaches for inducing T cell-mediated anti-tumor immune responses. In addition, we recently utilized a novel functional genomic approach to identify a new candidate tumor antigen that is overexpressed by the majority of pancreatic and ovarian cancers, as well as by up to 30% of all cancers. Mesothelin is one of a large category of identified tumor-associated, non-mutated self-antigens that is overexpressed by tumor cells relative to normal tissue. Although there are examples of the induction of T cell responses against this category of antigens, including mesothelin, currently employed vaccine approaches are not potent enough to overcome the mechanisms of peripheral tolerance that occur to these self-antigens. Our group has also been identifying signals within the tumor's micro-environment that are referred to as immune checkpoint inhibitors that inhibit effective antitumor T cell activity. These signaling pathways include B7-H1, B7-H4, and HVEM. We have been using relevant animal models to understand these pathways and for identifying the most potent combinatorial vaccine approaches that can overcome these mechanisms of peripheral tolerance, locally within the tumor's micro-environment, and thus be worthy of clinical testing. Through an NIH NCDDG grant, we have been developing the immunologic reagents (antigen peptides, tetramers, mesothelin targeted vaccines, and antibodies that inhibit these immune checkpoints) and testing these mesothelin-targeted vaccine approaches, in two mesothelin-expressing, murine tumor models, a pancreatic and ovarian tumor model, respectively. We have utilized these two mouse models to evaluate and develop more potent antigen-specific vaccine strategies combined with immune checkpoint inhibitors, that can overcome tolerance to tumor-associated self antigens. The development of these new biologic agents is based on a four step process: 1) Individual vaccine strategies under development by our group are first optimized for a number of parameters using transplantable pancreas and ovarian tumors. Each model provides unique opportunities to understand local T cell tolerance in the tumor's micro-environment. 2) Baseline immunologic effector functions are measured as an additional parameter for identifying the most potent vaccine approaches. 3) Optimized vaccine approaches are then compared head-to-head for the ability to eradicate naturally developing hepatic metastases (pancreatic tumor model) and peritoneal metastases (ovarian tumor model). 4) Based on data from the *in vivo* studies and the measured immune parameters, a targeted evaluation of potential synergies between different vaccines and immune checkpoint inhibitors are evaluated using potency against hepatic pancreatic tumor metastases and peritoneal ovarian tumor metastases as the final outcome.

Keywords: vaccines, immune checkpoints, pancreatic cancer